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CONCEPTS AND PROBLEMS CONCERNING THE ELECTRON MICROSCOPY OF PLANT VIRUSES[1]

Mark A. Stahmann and Paul Kaesberg

SUMMARY

The resolution of present day electron microscopes is better than 10 Angstroms but the full resolution cannot be realized with biological materials because contrast is insufficient. Plant virus specimens can be prepared in a number of ways. The spray-droplet method is noteworthy because quantitative results are obtainable. The freeze-drying method allows specimens to be prepared free from the distorting effects of surface tension. It is possible to obtain thin sections of plant tissues which may give information concerning virus reproduction. Future applications and limitations of the electron microscope are discussed briefly.

It is appropriate that a discussion of the electron microscopy of viruses should begin with the word "concepts", for it is in our conception of the nature and the appearance of viruses that the electron microscope has made its greatest contribution to virology. It has been demonstrated repeatedly that many fields of scientific investigation have made their most rapid advancement following the introduction of new methods or instruments that extended the limits over which natural phenomena could be observed and recorded. Just as the light microscope made a major contribution to bacteriology when it extended the limits of visual observation so that the bacteriologist could see and visually study the bacteria with which he worked, the electron microscope has extended our limits of observation so that the virologist now can observe the infectious virus particles with which he is concerned. And in time, it may well be established that the electron microscope has made a contribution to virology comparable to that of the light microscope to bacteriology.

Although the small bodies from lesions of a pox infection were observed in the light microscope over 50 years ago, it was not until the advent of the electron microscope that anyone was able to see a plant virus. In 1939 Kausche, Pfankuch, and Ruska (10) obtained the first pictures of particles of tobacco mosaic virus (TMV). Their pictures showed that the particles were slender and elongated, confirming the hypothesis of Takahashi and Rawlins who had come to a similar conclusion from physical-chemical evidence. As Williams has stated in his excellent review (27), this first observation clearly demonstrated that an important contribution of the electron microscope would be its ability to provide at a single glance confirmation of morphological conclusions secured indirectly and more laboriously by other methods.

In 1941, Stanley and Anderson (20) obtained electron micrographs of tomato bushy stunt virus and of tobacco necrosis virus and showed that they had a circular form. In 1945, when Williams and Wyckoff developed the shadow-casting method, it became possible for the first time to measure the dimensions of particles in a direction normal to their substrate. These authors (22) demonstrated that the thickness of tobacco mosaic virus was equal to its width and Price, Williams, and Wyckoff (14) similarly showed that the thicknesses of bushy stunt virus and of southern bean mosaic virus were nearly equal to their diameters. As the resolving power of the electron microscope was improved, a more detailed picture of viruses emerged. Williams (23) in 1952 succeeded in obtaining very small fragments of broken TMV and when one of these fragments was seen lying with its axis normal to the substrate it sometimes appeared to be hexagonal. Thus our present concept of tobacco mosaic virus is that of a long, slender rod having a hexagonal cross section and dimensions of about 150 by 3000 A. Williams (25) and Steere (21) have examined frozen-dried preparations of tomato bushy stunt virus and tobacco ringspot virus at high resolution and find that in outline they frequently have straight sides and often show a hexagonal contour. It is not yet possible, however, to give a detailed picture of their 3-dimensional structure.

Methods for observing viruses in their host cells have now become available through the development of techniques for cutting ultra-thin sections. It should thus be possible to investigate changing forms associated with virus growth in the cell. Only a start has been made in this direction and the number of papers thus far has been disappointingly small. However, Black, Morgan, and Wyckoff (5) succeeded in obtaining sections of tobacco leaves that showed the presence of TMV and that also exhibited other differences from normal plant tissue.

In this short introduction, we have mentioned the part the electron microscope can play, and have briefly surveyed some of the more notable events in

[1] Invitational paper presented August 26, 1954 at the Symposium on Concepts and Problems in Virology at the 46th Annual Meeting of the American Phytopathological Society in Estes Park, Colorado.

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its application to the study of plant viruses. In succeeding sections, we will discuss some of the future possibilities and limitations of these investigations and will describe some of the studies with which we are concerned.

RESOLUTION OF THE ELECTRON MICROSCOPE FOR BIOLOGICAL SPECIMENS.—The resolution limit of present-day electron microscopes is known to be slightly less than 10 A. The limitation is almost entirely due to spherical aberration in the objective lens. It is an obstacle of fundamental importance and is expected to remain with microscopes for a long time. However, the resolution limit for untreated biological specimens is at least an order of magnitude worse, because of the fact that the electron optical density of biological particles is so low that they cannot be seen if they are less than about 100 A thick. To put it slightly differently, the number of electrons removed from the electron beam by a biological particle with a diameter of 100 A is small compared to the number of electrons removed by the collodion film ($\geqq 300$ A) on which the particle rests and the contamination film (several hundred) that deposits on the sample when it is irradiated by the electron beam. The origin of the contamination film is not completely settled but it apparently is produced in all electron microscopes thus far constructed. Objects thicker than about 1000 A remove such a large proportion of the electrons from the beam that the objects appear opaque and thus no internal structure is visible. Even in the case of plant viruses, many of which are in the ideal size range of 100-1000 A, no internal structure has been reported.

It is clear that the contrast of biological materials must be enhanced in order to make full use of the resolving power of the electron microscope. Staining techniques in electron microscopy are relatively undeveloped. It is only the heavy atom stains that are effective and usually osmic acid and phosphotungstic acid are the materials that have been used, although uranyl nitrate and iodine have been suggested. Some protein fibers have been stained successfully but as yet there appears to be no evidence in the literature that plant viruses have been or can be stained.[2] This is an important and much neglected field of investigation and merits further study.

An almost universally used alternate method to increase contrast is shadow-casting. In this method the specimen is placed in an evacuated evaporating chamber and a thin film of some heavy metal is deposited by evaporation obliquely onto the surface of the sample. The usual metals are platinum, palladium, or uranium. The amount of evaporated material varies from point to point because of the contour of the substrate and the specimen lying on it. It is obvious that the top surface of the sample will be delineated with high contrast while the interior will be somewhat obscured. Thus, the shadow-casting method is exceedingly useful in helping us to see small particles but it should not be used indiscriminately for visualizing fine structure in large particles. Using the shadow-casting method, particles having a linear dimension as small as 20 A have been detected. These have been, however, the very elongated particles of nucleic acid (24) which could be identified although the collodion film upon which they rested had granules larger than 20 A.

ELECTRON MICROSCOPY OF PURIFIED PLANT VIRUSES.—The first application of the electron microscope to the study of a new plant virus is apt to be for the purpose of identifying it morphologically. The time-honored procedure consists in showing that characteristic particles are present in large numbers in an infectious extract prepared from diseased plants but that these particles are absent in similar noninfectious preparations from healthy plants. The ease with which this may be demonstrated depends upon the physical and chemical characteristics of the particles, particularly their stability during purification and their concentration in the plant. The further step, which consists of showing that the characteristic particles are indeed the virus, is much more difficult and has been all but ignored in most plant virus studies. Much of the reason for this difficulty lies in the lack of sensitive and accurate ways to check infectivity.

Sometimes infectious extracts can be prepared very simply by placing the drops of cell sap, forced out of cut stems or petioles by water pressure, onto microscope screens as suggested by Johnson (8), drying, washing to remove soluble salts, and shadowing. This method allows one to examine a sample of the contents of the vascular system and under these conditions, when virus particles are present, they often are associated with somewhat less extraneous material than if the whole tissue is ground and extracted.

The more commonly used technique for preparing plant viruses for study with the microscope consists of grinding infected plant tissues, squeezing out the sap, and extracting the pulp with a buffer. The combined sap and extracts are then subjected to a series of low- and high-speed centrifugations to separate the cell debris, smaller proteins, and other substances from the high-molecular weight virus particles. Alternatively, chemical separations may be used. The drops from a suitably diluted portion of the purified virus solutions are placed upon a microscope screen and dried.

When we applied Johnson's method to pea plants infected with the Wisconsin pea streak virus, pictures were obtained which showed that numerous curved rod-like particles were present in sap from infected plants (Fig. 1, A), but not in that from healthy plants. By differential centrifugation, we also isolated virus from plants just beginning to develop symptoms and from severely infected plants. The particles from the former were somewhat longer than those obtained by the water pressure method, whereas the particles from the plants with a severe infection were aggregated into very long strands (19). When solutions of these virus preparations were allowed to stand *in vitro* the end-to-

[2] C. E. Hall (unpublished) has recently succeeded in staining tobacco mosaic virus and bushy stunt virus.

end aggregation continued and even longer strands developed, some of which extended for many microns. The width of the particles in all preparations was the same. Thus, the Wisconsin pea streak virus, which is

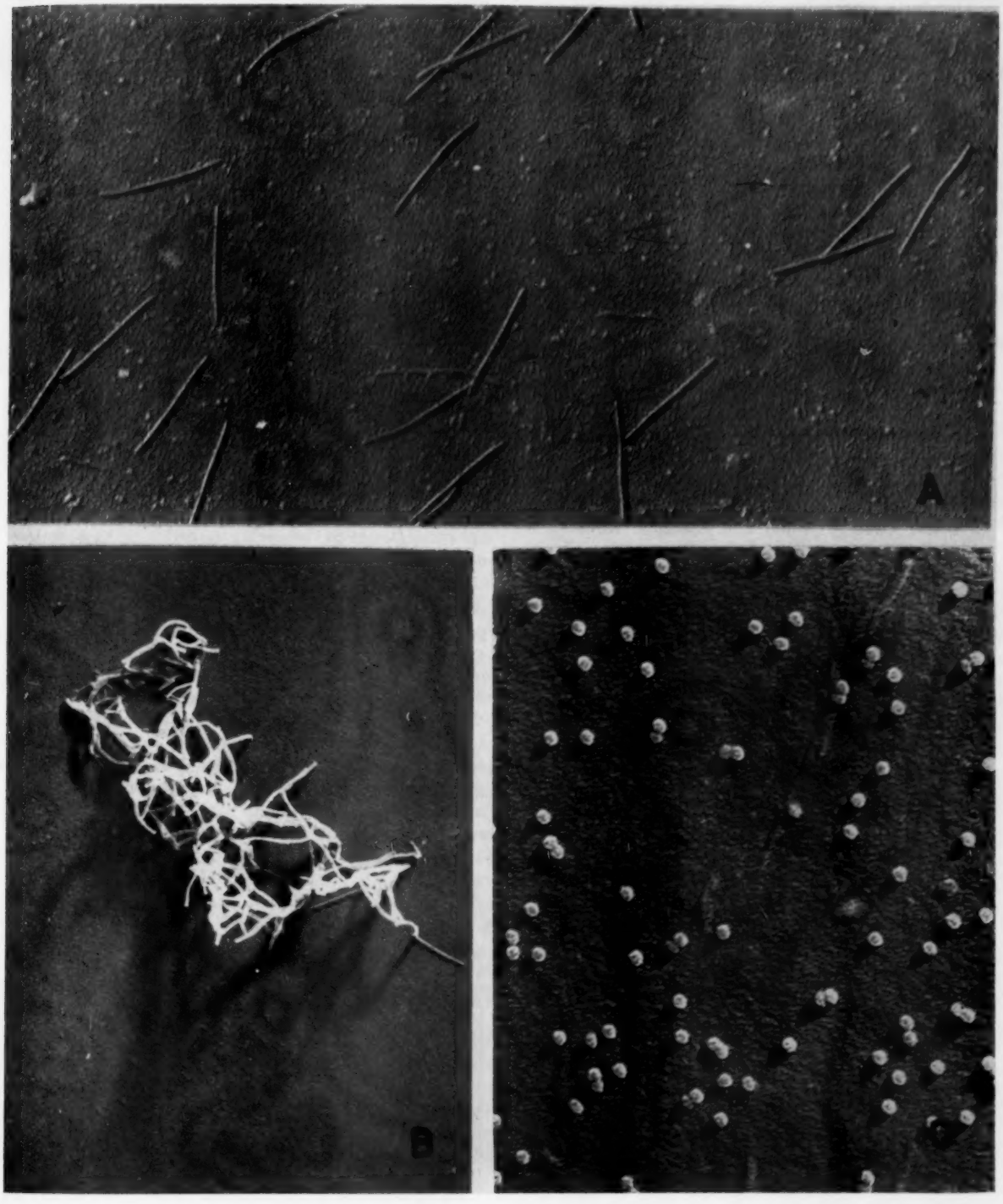

FIG. 1. A) An electron micrograph of Wisconsin pea streak virus prepared by Johnson's method. Magnification 28,000×. B) An electron micrograph of frozen-dried tobacco mosaic virus. Many of the particles are sticking up from the substrate and cast long shadows. Magnification 33,000×. C) An electron micrograph of frozen-dried turnip yellow mosaic virus. Magnification 60,000×.

initially produced as short flexible rods about 7000 A in length, aggregates very readily into longer flexible particles both within the plant and in the test tube.

This work emphasizes that certain plant virus particles may undergo changes in form as infection advances. These changes often are the result of aggregation of the virus particles. Another possible change is a reaction with other proteins of the plant cell to produce a complex consisting of the virus nucleoprotein and plant proteins. It has been demonstrated that such interactions readily occur *in vitro* with tobacco mosaic virus and the virus inhibitor protein from spinach (11) or from phytolacca (9) to produce a complex that is not infective. That a similar reaction could also occur *in vivo* in certain cases would seem very likely. Such a reaction may explain the beaded appearance that has often been observed with crude preparations of tobacco mosaic virus. This irregular beading would seem to be due to the attachment of plant proteins to the surface of the virus particle. Hence, in working with impure virus preparations, one must always consider the possibility that virus complexes are observed instead of individual virus particles.

Quantitative methods.—In general it is not possible to get quantitative results from microscope specimens deposited in the form of a large droplet. As the droplet dries, surface tension and chemical forces move and rearrange small particles in an unpredictable way so that it becomes infeasible to select representative areas to photograph. Backus and Williams (4) have overcome some of these difficulties in a very original way by depositing their specimens in the form of an exceedingly fine spray using either an atomizer or a high velocity spray gun. Each droplet is sufficiently small to be photographed in its entirety and the contents of each are found to be entirely representative of the bulk suspension. Since volatile suspending media (e.g. distilled water, ammonium acetate in water, ammonium benzoate in water) are used, no washing is required. Polystyrene indicator spheres in known concentration are in many cases added to the suspension since their number in a particular droplet is an immediate measure of the droplet's volume. Two other methods that give uniform dispersion and that are thus susceptible to quantitative interpretation deserve mention. Sharp and his collaborators have deposited specimens onto films placed in a centrifuge (17). Hartman and his co-workers disperse their particles in a protein surface film (7).

Freeze-drying of plant viruses.—All the methods for preparing virus specimens that we have described so far have the common difficulty that the water surrounding the virus particles must be evaporated. As the evaporation proceeds, the water surface film exerts a tremendous pressure on particles as small as viruses and distorts them. Anderson (2) has calculated that this distorting pressure may exceed a ton per square inch. Most of this distortion takes place in the last stages of drying as the water film, in maintaining a minimum area, presses the particles against the substrate. Thus red blood cells, bacteria, and spherical viruses appear flattened as a result of air-drying and are then, in effect, seen in only 2 dimensions. Williams (26) has shown that by properly freezing the solution and removing the ice by sublimation, this destructive phase change is avoided, and even such fragile particles as red blood cell ghosts now appear undistorted. He has carefully defined many of the conditions for adequate freeze-drying and has applied the method to a variety of biological materials.

We have developed a similar but somewhat simplified arrangement (15) which allows us to prepare a considerable number of specimens in one operation. Figure 2 illustrates some of the features of our modification of the freeze-drying method. The virus suspension is sprayed with a spray gun and guide tube onto the specimen film supported by a microscope screen on a large specimen block in contact with liquid air as shown at the right. Under these conditions the microdroplets are frozen extremely quickly. The blocks with the specimen screens are then transferred to another chamber and the ice is removed by sublimation at a temperature well below the melting point. Figure 2 also shows the sublimation assembly and the cold trap, all contained inside a cold chest. This arrangement allows the transfer of the frozen mounts from the spraying Dewar to the drying assembly without danger of melting.

Figure 1, B shows a group of tobacco mosaic virus particles prepared by this method. The individual particles are clearly resolved and extend to a considerable height above the collodion film as the long shadows indicate. It is apparent that no water surface film existed and that the distorting forces were small. With concentrated suspensions, the particles are often seen in the form of clusters as shown here. This results from the fact that as the ice leaves the frozen droplet, the small virus particles are knocked about by thermal agitation and must eventually leave the specimen or come to rest by adhering to the substrate film or to another virus attached to the film.

An example of a spherical plant virus is given in

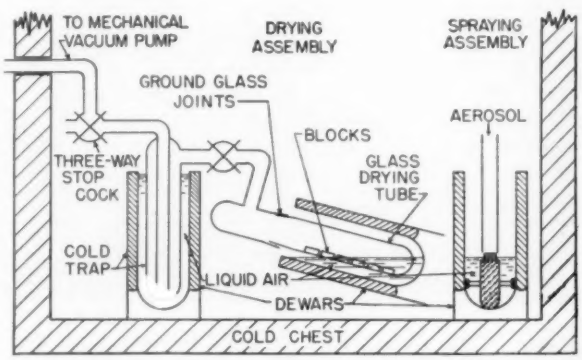

FIG. 2. A diagram showing the apparatus used to prepare frozen-dried specimens for electron microscopy.

Figure 1, C which is an electron micrograph of frozen-dried turnip yellow mosaic virus. These particles usually are not quite circular in contour and sometimes appear to have flat sides or to have a slight bulge on one side. The particles of turnip yellow mosaic virus are of particular interest because, as Markham and Smith (12) have shown, they exist as 2 very similar forms. One is infective and contains nucleic acid, whereas the other is noninfective, lacks nucleic acid, and consists solely of protein. They are nearly the same size and shape and are thus far indistinguishable in the electron microscope. Following a procedure developed by Markham (13), we recently have separated the 2 components for study by small angle X-ray scattering. This study (16) showed that both the virus and the protein in solution have a diameter of 280 A and that the protein component is a water-filled spherical shell with a ratio of inner to outer radius of about 0.75. It would appear that the infective virus is formed when the water in the center cavity of the protein is replaced with nucleic acid. It would be of considerable interest if this structure could be observed by electron microscopy.

The particles in frozen-dried preparations of squash mosaic virus[3] resemble those of turnip yellow mosaic virus and also show deviations from a circular contour. At high magnification the contours appear to be hexagonal (Fig. 3, A). As with turnip yellow mosaic virus, these suspensions contain 2 principle centrifuge components, but we have not yet succeeded in obtaining pure separated components for identification and study.

The fine structure of viruses.—Since it has not been possible to see any internal detail in plant viruses, other methods must be sought to obtain information concerning their structure. One way consists in breaking the virus into smaller fragments and examining them. Anderson (1) first succeeded in such an experiment when he subjected bacterial viruses to osmotic shock and showed clearly that the virus head is a thin membrane which prior to the fragmentation probably held the virus nucleic acid. Williams (25), in a very elegant experiment, separated the nucleic acid from an individual frozen-dried bacterial virus by rewetting the dried particle. Since the parts of the particle remained nearby and could be examined, this probably could be called a dissection—undoubtedly the smallest on record.

We have investigated in some detail the breaking of tobacco mosaic virus particles in the freeze-drying process (15). Figure 3, B shows 2 of these rods broken into pieces by this technique. It is to be noted that the small pieces are not free to move and that their original in-a-line arrangement is retained, so here also the parts from a single particle are identifiable. On close examination, small amounts of material sometimes can be seen between broken pieces. This may result from a pulling apart of the particle; it could be nucleic acid released from the interior.

We do not know the exact nature of the underlying forces involved in the breaking of tobacco mosaic virus particles but it probably is related to the length of time the virus is surrounded by ice in an environment of sublimation. We have observed breaking in several strains of tobacco mosaic virus and in potato-X virus, but not in bacterial flagella. The pressure in the system during sublimation is directly related to both breakage of virus particles and to the appearance of good 3-dimensional virus masses. When the ice was sublimed at −22°C at 0.03 mm of mercury, little breakage and the best resolution of individual rods in the masses was obtained. Individual rods were found broken with fairly good resolution of the masses at 1 mm pressure. At 11 mm we found no 3-dimensional virus masses and many broken rods in-a-line as well as flattened masses of broken rods.

STUDIES OF CELL SECTIONS.—One of the most interesting applications of the electron microscope to virology is the study of virus particles as they are formed within the cell. In such a study it is desirable to preserve the *in vivo* localization and morphology of the developing virus particles. Since we cannot observe living cells in the electron microscope, we must use either thin sections of fixed material or whole cells in a dehydrated condition. In collaboration with Drs. Skotland and Hagedorn (18), we recently attempted a study of the Wisconsin pea streak virus similar to that of Black, Morgan, and Wyckoff with TMV, but we were unable to locate the thin virus particles in leaf or stem sections of infected pea plants. In checking our technique, we made sections of tobacco leaves infected with tobacco mosaic virus. We would like to discuss some of these as an illustration of a study of the intracellular growth of viruses.

The sectioning of cells for electron microscopy is fundamentally the same technique as that used in cytology, histology, and pathology, although it is modified to meet the need of extreme thinness. The technique consists primarily of fixing of the tissue with buffered osmium tetraoxide or a formalin-aceto-alcohol mixture, dehydrating through graded alcohols, immersion in a methacrylate monomer which is polymerized, and then ultrathin sectioning with a special microtome. The microtome may be an ordinary rotary type in which the feed mechanism has been modified so that it is advanced mechanically at a reduced rate or by thermal expansion. The cutting may be made with carefully sharpened steel knives, or with cutting edges selected from plate glass parallelgrams broken at an angle.

Examination of over 200 sections of cells revealed that the most characteristic difference between normal and infected cells was the appearance of many small dense bodies dispersed throughout large portions of the virus-infected cell, whereas in the healthy cell these bodies are found only within the chloroplasts. These dense bodies are probably grana, and possibly

[3] Rice, R. V., Lindberg G., Kaesberg, P., Walker, J. C., and Stahmann, M. A. (unpublished).

other particles, released into the cytoplasm by the breaking of chloroplasts following the virus infection. Other sections showed amorphous material about these particles and about rod-shaped structures resembling virus. Figure 3, C shows a section through a severely infected cell. Here the cell wall can be seen in each

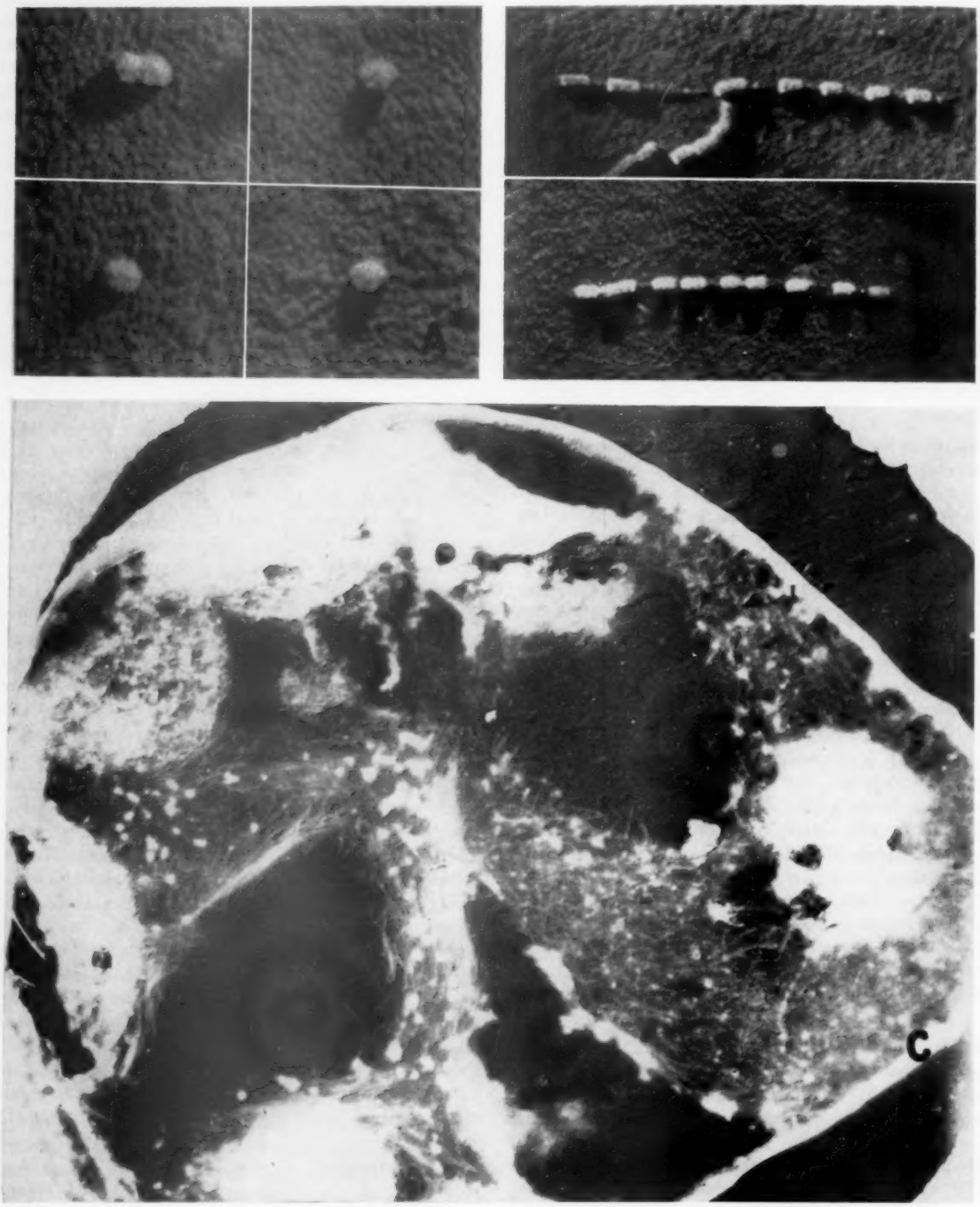

FIG. 3. A) Four high magnification electron micrographs of frozen-dried squash mosaic virus showing that the particles sometimes seem to have a hexagonal contour. Magnificatio n 150,000×. B) Two electron micrographs of tobacco mosaic virus broken in-a-line by freeze-drying. Note that there is m aterial between some of the pieces. Magnification 100,000×. C) An electron micrograph of a section from a leaf of an infected tobacco plant. Note the presence of the small dense granules. Magnification 10,000×.

corner with several chloroplasts adhering to it. Some of the chloroplasts seem to be intact, others appear to have disintegrated. Near the center of the picture many of the small dense spherical bodies can be seen. Figure 4, A shows at higher magnification a number of the dense particles surrounded by rod-shaped structures which we believe are TMV. Virus was never seen in the nuclei.

The consistent association of virus particles in cell sections with these small dense approximately spherical particles would suggest that virus infection results in a breakdown of some of the chloroplasts. The yellow chlorotic symptoms commonly associated with infection with tobacco mosaic virus and many other plant virus infections would support this view. It might suggest that the grana or other particles with enzymatic activity in the chloroplasts actually are utilized in the synthesis of new virus nucleoprotein. Such a mechanism would be consistent with the results we have obtained.

We have presented these results in some detail and have speculated on the interpretation of the pictures with the hope that it might stimulate an examination of plant virus material in thin sections. We feel that further application of the electron microscope to this type of study will contribute materially to our understanding of the development of viruses within the infected cell.

FUTURE PROGRESS IN PLANT VIRUS STUDIES.—Thus far we have tried to illustrate how the electron microscope has contributed to our present concepts of the structure of plant viruses. Now, we would like to discuss some of the problems and questions to which the electron microscope may contribute in the future.

One of the first questions that might be asked is how much more resolution can we expect from future electron microscopes. The theoretical resolution limit fixed by the wave length of the electron beam is several hundred times below that of our best electron microscopes. However, we cannot expect that the resolving power of the electron microscope will improve appreciably until new methods are discovered to reduce the spherical aberration in the microscope lens systems. We can, however, expect significant improvement in contrast as new photographic emulsions are introduced and better methods are found to eliminate scattered electrons from the final microscope image. Whether or not we can see plant viruses better will depend much more on our success in eliminating contamination both in the microscope itself and in the shadowing units. This contamination seems to arise from oil and other organic molecules in the vacuum systems and is deposited as a thin film over the entire mount. The contamination arising in the shadowing unit is the more dangerous because it is shadowed along with the specimen and thus is easily visible in the microscope. Figure 4, B shows this contamination on a sample of tobacco mosaic virus shadowed with uranium. The rods on the left have the normal appearance of shadowed virus particles. However, the rods on the upper right were fortuitously lying with their axes in the direction of the shadowing so that they have been shadowed "end on" and can be seen only in outline. From the length of the shadow they cast it is obvious that they are rods of normal thickness. Nevertheless, they are difficult to see because the appearance of their surface is exactly the same as that of the substrate right next to them. Apparently a thin contamination film produced in the shadowing unit covers both virus and substrate and we see only the surface detail of this film.

As you realize, such improvements in microscope construction will be made by the physicists and by the engineers who build them. Now we would like to point out some of the applications that the biologists and the plant pathologists who use the microscope might profitably consider.

We have illustrated how a study of ultrathin sections of infected plant material may contribute to our understanding of where and how viruses are formed within cells. The virologists working with animal viruses already have applied the thin-sectioning technique to a study of virus development in tissue cultures. This may be a profitable field of investigation of plant virus development also, because the virus can be expected to advance more uniformly along the cells of a small, tissue-cultured group than in the massed cells of whole plants.

We know relatively little about the structure of plant viruses. It would appear possible to gain some insight into this if better methods of staining with heavy metal, or other electron scattering stains, could be developed. Stains specific for nucleic acids are needed, as well as better protein stains with high electron scattering properties.

The application of replicate techniques by Wyckoff (28) to the study of virus crystals and by Hall (6) to the study of the surface structures of protein crystals already has given us an insight into the beautiful orderly arrangement of the protein molecules in these crystals. This method could expand our knowledge of molecular growth and crystal structure. It might be feasible to apply the method to some of the larger virus particles themselves.

The application of quantitative electron microscopy for the determination of the number of particles in a preparation, for the correlation of number of particles with infectivity, and for the determination of the molecular weights of virus particles merits a wider application.

Further work is needed to determine the best methods for the preservation of 3-dimensional structures. More study of the freeze-drying method is needed to decide just what is the best way to spray, freeze, and dry virus specimens to preserve their original structure. The pictures already obtained by the present freeze-drying methods are so much superior to those obtained by the ordinary air-dry techniques that the

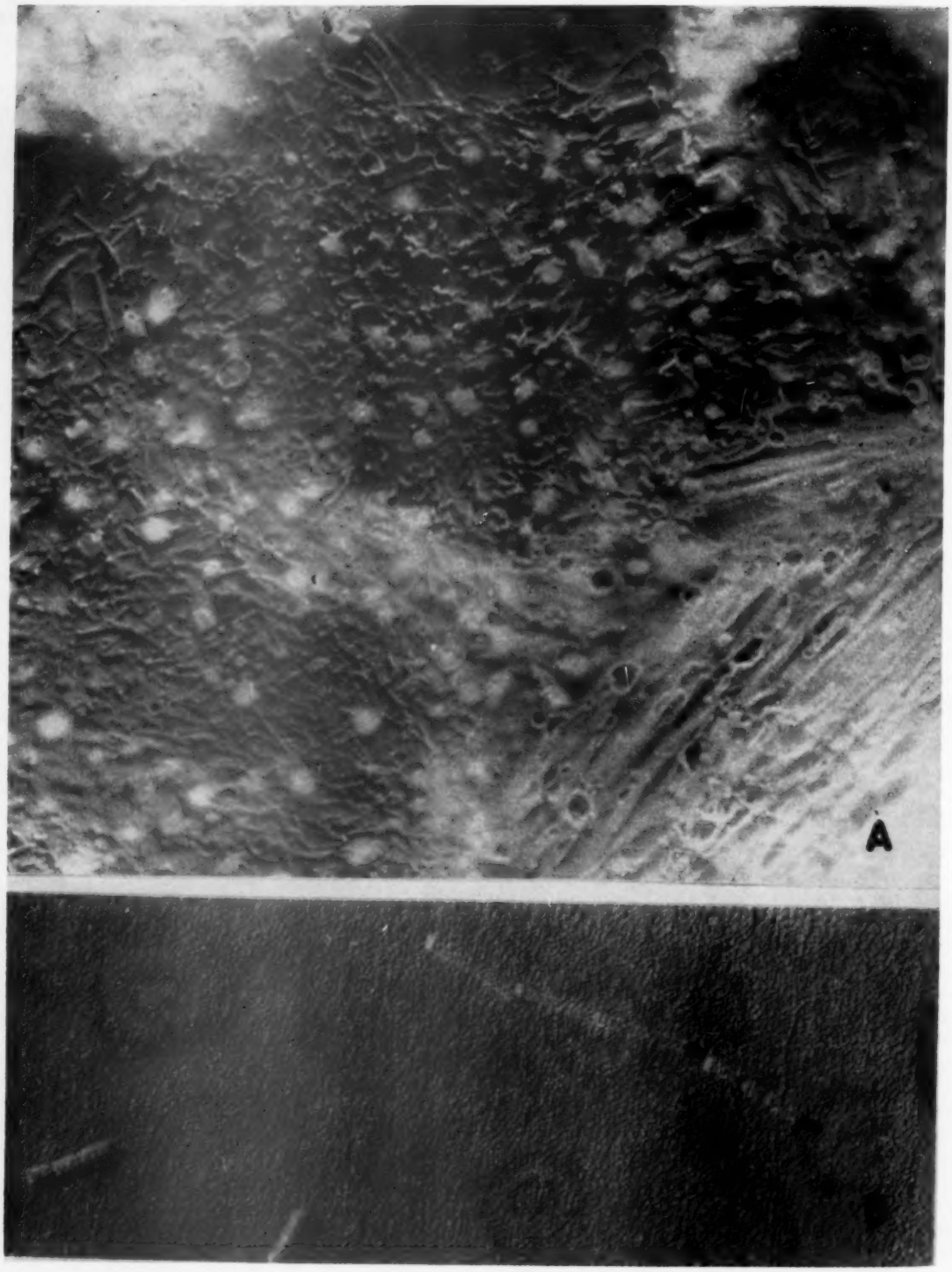

Fig. 4. A) A higher magnification view of a section like that of Figure 3, C. TMV rods as well as dense granules are visible. Magnifications 25,000×. B) An electron micrograph of TMV showing the presence of contamination. The rods on the upper right were oriented so that they were shadowed exactly "end on". They cast a shadow having the length expected for a normal rod; however, the particles are barely visible because their surface has the same appearance as the substrate on which they rest. Magnification 125,000×.

latter should not be used for serious study of virus morphology.

The critical-point method of Anderson (3), in which fixed specimens are brought into liquid carbon dioxide and freed of it by allowing it to escape at a temperature above its critical point, merits application to plant virus studies. Anderson has succeeded in a truly remarkable preservation of 3-dimensional structure with a number of biological materials.

In conclusion, we would like to emphasize that the electron microscopy of viruses is still very young. It was only a relatively few years ago that a virus nucleoprotein was photographed for the first time. We expect a continued rapid growth of knowledge of the morphology and of the formation of viruses as this new powerful tool finds wider application by virologists.

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CONCEPTS AND PROBLEMS CONCERNING THE ASSAY OF PLANT VIRUSES[1]

Russell L. Steere

SUMMARY

Although infectivity of plant viruses is quite definitely associated with, and inseparable from, the presence of characteristic particles, one cannot say unequivocally that one of these particles is an infectious unit. Furthermore, we are unable to determine the total number of infectious units in a sample. With these 2 considerations in mind, a review of the use of both biological and nonbiological procedures that have been described for the assay of plant viruses is presented. Two fundamentally different biological assay methods have been employed. One involves the mechanical inoculation of systemic or local-lesion host-plants. The other depends upon an insect vector to transmit the infectious agent to the plant. The nonbiological methods used to assay plant viruses consist of serological procedures, the electron-microscope particle-count procedure, purification followed by dry-weight determination, chemical procedures, a spectrophotometric procedure, a colorimetric procedure, and a procedure based upon the dilution end-point of stream double refraction. These methods are discussed individually and unique advantages or disadvantages of each are reported. A table is presented for comparison of the various procedures that have been used to assay tobacco mosaic virus. This table includes a description of that which is actually measured by each procedure. Estimates of the number of characteristic particles and of the approximate dry weight of purified concentrates of these particles required for an assay also are included. The anticipated error of each assay method is presented.

The combined use of 2 or more procedures is discussed and a method is described whereby it has been possible to obtain a lesion for every 50,000 characteristic particles of tobacco mosaic virus and 1 lesion for every 120,000 characteristic particles of tobacco ringspot virus.

A brief discussion is devoted to the comparison of plant-virus assays with those of animal viruses and of bacterial viruses (bacteriophages). The much closer correlation of infectivity with the characteristic particles, essentially 1 infection for every particle, for some animal and bacterial viruses is pointed out.

The major remaining problem relative to plant-virus assays, that of obtaining conclusive evidence that an infectious unit is a single characteristic particle, is discussed and several methods are suggested for its possible solution.

Our ability to assay the quantity of virus present in selected samples of infectious material is the foundation for much of our knowledge concerning the properties of viruses. Assays permit us to compare one virus suspension with another, to prepare purified virus suspensions and correlate infectivity with the characteristic particles found therein, and to examine the infectious agents by various physical and chemical means. Without suitable assay methods we could not determine whether the characteristic particles found only in infected tissue include in their numbers the infectious agents or are merely products of these agents or of the infected tissue. It is well, therefore, to consider 2 fundamental problems whenever we wish to conduct an assay of a plant virus preparation: 1) what is the infectious unit, and 2) how can one determine the number of infectious units contained in the sample?

Unfortunately, it is not yet possible to obtain an unequivocal answer to either of these questions where the infectious material involved is a plant virus. In attempts by various workers to determine the nature of the infectious agents, some half dozen or more plant viruses have been highly purified as evidenced by the fact that the resultant suspensions have been shown to be homogeneous by the various tests available for checking this condition. Notable examples of such plant viruses are tobacco mosaic virus (TMV) (49, 50, 93, 102, 103), tomato bushy stunt virus (6, 48, 54, 68, 107), and southern bean mosaic virus (33, 71, 72, 73, 76, 78). These purified preparations are highly infectious and each has been found, by electron microscopy, to consist of a suspension of particles of uniform size and shape (81, 82, 108, 120, 121, 122, 123). The particles are characteristic for any one virus regardless of the host from which they are isolated (36, 58, 106, 107, 109) but for different viruses the particle morphology may differ. Rod-like particles, spherical particles, polyhedrons, and ellipsoidal bodies have been observed.

For convenience, those particles which are associated with infectivity for each virus will be referred to as "characteristic particles". They will not be called "virus particles" nor "infectious agents" in this discussion because the correlation between number of characteristic particles in a virus suspension and the number of infections that has been obtained is not good enough to permit one to assume that an infectious

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unit is a single particle of the size and shape observed.

To be sure, experiments have been conducted with a view toward determining whether the characteristic particles are or are not the infectious agents and much information has been assembled which favors the assumption that the 2 are identical. Stanley (100, 101, 104, 105, 106), in his classic work on the purification of TMV, conducted a number of such experiments and found that procedures that removed or destroyed the characteristic particles also always removed or destroyed infectivity. Lauffer (51) and Epstein and Lauffer (33) have suggested and employed the use of various parameters to correlate infectivity with the characteristic particles. They have found that infectivity invariably is associated with components that behave in the same manner as do the characteristic particles when they are subjected to physical forces. Chester (25) presented serological evidence that the antigen and virus agent are identical. He found that virus suspensions subjected to heat, acid, or alkali treatments severe enough to cause loss of infectivity had lost a corresponding proportion of their ability to precipitate antibody. He also found that virus from different host plants has a precipitin capacity proportional to its infectivity.

Lavin and Stanley (55) in their study of the ultraviolet absorption spectrum of TMV found a maximum at 2650 A, the same wave length that Duggar and Hollaendar (30) previously had observed to be most lethal for this virus.

Rochow, Ross, and Siegel (89) recently have obtained experimental results that suggest a direct correlation between the number of particles of potato virus X and the number of local lesions obtained on local-lesion host plants. Samples found to differ by a certain amount in infectivity were found to differ a corresponding amount in the number of countable particles.

The evidence presented thus far points to the very close association of infectivity with characteristic particles for plant viruses. Other evidence suggests that 1 infectious unit is capable of initiating an infection and that multiple infections are not necessary. In 1934, Kunkel (47) reported that single necrotic lesions produced on leaves of *N. langsdorffii* inoculated with a mixture of equal parts of two strains of TMV contained usually 1 or the other strain but seldom both. Lauffer and Price (52) repeated this experiment with some variation and obtained similar results. They analyzed their data and found it in satisfactory agreement with the theory that 1 infectious unit is capable of causing an infection. They also examined data drawn from the literature and found that "all of the data examined tended to be consistant with the theory that the quantitative response at various levels of dosage was due primarily to the chance occurrence of at least one infectious unit in the dose". They report further that "the data excluded entirely the possibility that the response at various levels is due to the chance occurrence of a minimum of two or more units." Additional evidence that 1 infectious unit is sufficient to cause an infection is found in the relative ease with which strains of a virus can be isolated through local lesions (43).

I wish to stress the fact that experimental evidence leads one to believe that an infectious unit is a single characteristic particle and that the only reason for not using the term "virus particle" in this discussion is that only a small number of infections can be obtained with an inoculum containing millions of these particles. Until infectivity can be associated with fewer than 100 characteristic particles we will do well to be cautious about assuming that all the characteristic particles are infectious agents. If only 1 particle in 50,000 is infectious it hardly can be claimed that the observed homogeneity of the virus preparations is significant as far as the infectious agent is concerned. We certainly could not expect to distinguish unequivocally, by any nonbiological procedure, the presence of small infectious particles in a preparation containing 50,000 times as many noninfectious particles of approximately the same size and shape.

Not only are we unable to identify with certainty the infectious agents for plant viruses, but as previously stated we also are unable to determine the number of infectious units in a sample. Only relative infectivities of 2 or more samples can be determined. It is obvious to anyone who has made a biological assay of plant-virus preparations that very few of the infectious units are able to produce infections under conditions currently employed. From experimental evidence (17, 41) and from working with plant-virus infections, one gains the impression that infection with a plant virus usually occurs when an infectious unit enters a cell at the time an injury is produced which is so slight that the normal functions of the cell are not significantly disrupted. Excepting those that involve the use of insect vectors or grafting, the procedures currently in use for the biological assay of plant viruses depend upon mechanical inoculation in which some applicator such as a glass spatula, a brush, a cheesecloth pad, a pot label, or some other object is dipped into the inoculum and then rubbed over the surface of the leaf of a host plant. Depending upon the virus-host relationship used, readings are made in terms of the percentage of plants that become infected, the number per leaf of local lesions (chlorotic or necrotic spots that appear at the site of each infection) (40), or the number per leaf of starch-iodine lesions (localized infections that contain more starch than the surrounding tissue and thus stain more heavily with iodine) (42). Certainly one could not expect a very high percentage of the infectious units present in an inoculum to cause infection where such a system of inoculation is employed. Only a small percentage of the infectious units could come in contact with the cells at the time of inoculation; most of them would be found elsewhere in the film of inoculum on the leaf surface. An equally

small percentage of those that do make such contact would be in a position such that they could enter a cell when a slight wound is produced. It is not surprising, then, that the ratio of the number of infections obtainable to the number of characteristic particles in the inoculum is poor, even if it is assumed that every particle is an infectious unit.

All available evidence supports the theory that most characteristic particles of the more stable plant viruses are also the infectious units. On this theory, our inability to obtain infection for essentially every particle is thought to be due to our very inadequate biological assay procedures. Rappaport and Siegel (83, 84) suggest a different theory. They report the results of experiments involving the use of rabbit antiserum and ultraviolet irradiation for the inactivation of TMV preparations. They believe their data suggest that only a minute fraction of the total number of characteristic particles, as ordinarily prepared, is capable of initiating infection. This theory certainly simplifies the problem of explaining our inability to obtain an infection with fewer than 50,000 characteristic particles. We know, however, that our inoculating procedures are extremely wasteful of virus, and it is believed that the procedure by which a lesion was obtained for each 50,000 particles (111), reported elsewhere in this paper, can be improved to a point where 1 lesion can be obtained for each 1,000 or fewer particles. If this can be accomplished, many more infectious particles will be shown to be present than the number suggested by the theory of Rappaport and Siegel.

ASSAY PROCEDURES.—We have observed that a direct measure of the number of infectious units in a plant-virus preparation is impossible to obtain with current procedures. One might therefore, wish to ask these questions concerning the assay of viruses: 1) What methods have been employed for the assay of plant viruses? 2) What does each method actually measure? How reliable are the results? And what are the special advantages and disadvantages of each? 3) How do plant-virus assays compare with animal-virus and bacterial-virus (bacteriophage) assays? 4) What procedures might conceivably be developed for the improvement of plant-virus assays with the ultimate aim of obtaining an infection for every infectious particle?

The various methods used for the assay of plant viruses fall into 2 groups: those that measure biological activity and those that depend upon other properties (chemical, physical, or morphological) of the characteristic particles. Each of several procedures has been used at one time or another with the aim of measuring either the presence of virus or the quantity of virus or the number of characteristic particles in a preparation. Each has its limitations as well as advantages and the following discussions are designed to point out these differences. Table 1 illustrates the comparative capabilities of the different procedures for the assay of TMV. In the preparation of this chart, the assumption was made that the characteristic particle of TMV is 300 mμ in length and 15 mμ in width (123) and weighs 8.2×10^{-17} g (122), and purified preparations have been assumed to contain particles of this size only. For viruses that have larger or smaller characteristic particles or particles of a different density the values shown in the table may not apply.

Biological assays.—Biological assay procedures currently employed can be used to measure only the relative infectivities of 2 or more samples. They must be relied upon, however, for the association of infectivity with the material that is examined by all nonbiological assay procedures. It is extremely important, therefore, that biological assays be made as reliable as possible.

1) *Systemic infection.*—Of the procedures in use today for the biological assay of mechanically-transmissible plant viruses, 1 involves the use of host plants that become systemically infected. Serial dilutions of the test preparation and of a standard preparation are rubbed onto the leaves of many separate test plants. A record is then made of the number of plants that become infected with each dilution of the test sample and with each of the standard. From these records, it is possible to estimate the virus content of the test sample relative to that of the standard. Holmes (39) has discussed the use of this procedure for quantitative work with TMV. He reported that the number of plants required for the detection of various dilutions of a sample of diluted juice from a TMV-infected plant ranged from 30 to detect a dilution of 1/64 to 1350 to detect a dilution ½. This procedure is cumbersome and is highly subject to errors resulting from nonhomogeneity of the test plants. Unfortunately, there are many plant viruses for which no better biological assay procedure is yet known.

2) *Local-lesion assays.*—A fundamental advance in the biological assay of many plant viruses resulted from the discovery by Holmes in 1929 (40) that local lesions develop on some plants at each site of infection. With this first report of local lesions, Holmes suggested a procedure for quantitative studies with TMV. With the use of local-lesion hosts, it is possible to obtain more reliable information from a few plants than can be obtained with the use of 50–100 times as many systemic host-plants. Using local-lesion hosts, one can compare at least 2 and often more samples on the leaves of each test plant. This reduces considerably the errors due to inhomogeneity of the test plants.

Several leaves on a single plant may be used with a different preparation applied to each leaf, and some scheme such as the Latin square as employed by Youden and Beale (127) can be arranged so that each preparation appears equally often on each plant and at each leaf position. This reduces to a minimum errors due to differences in susceptibilities of different plants and of different leaves on a plant. A more reliable procedure is one involving plants with opposite leaves (often seedling legumes) in which the primary leaves are used. In such experiments 1 leaf can be

TABLE 1.—*Comparative data on the various methods that have been employed or suggested for the assay of tobacco mosaic virus. All figures are based on the values for purified preparations of the characteristic particles of TMV irrespective of whether or not highly purified preparations are needed for the given assay method*

Assay method	Item actually measured	Approximate quantity of purified preparation of characteristic particles needed for assay[a]				Approximate error[a]
		Dry weight per ml	Number of ml	Total dry weight	No. of particles	
Biological Assays		mg/ml		mg	$\times 10^6$	per cent
Mechanical inoculations to						
Local-lesion host	Relative infectivities of 2 or more samples	0.000001	5	0.000005	50	10
Systemic host	Relative infectivities of 2 or more samples	0.000001	5	0.000005	50	20-50
Nonbiological assays						
Serological Tests						
Precipitin (ring test)	Dilution end-point	0.5	0.01	0.005	50,000	20
	Quantity of precipitate	1.8	1	1.8	20,000,000	5
Red blood cell precipitin-test	Optimum antigenic units of standard and unknown	1.8	1.2	2.2	30,000,000	
Complement fixation	Amount of complement fixed	0.02	1	0.02	300,000	50
Particle count with electron microscope	Number of characteristic particles per ml	0.0001	0.01	0.000001	10	5
Dry weight of purified virus	Dry weight of purified material			0.0001	1,000	5
Chemical	Amount of N, P, or other chemical component in preparation			0.003	40,000	5
Optical Density	Amount of absorption at 2600 A	0.075	3	0.025 est	300,000	5
Colorimetric	Color intensity with Folin reagent	0.002	3	0.008	100,000	7
Critical dil. of stream double refraction	Dilution at which SDR disappears	0.003	10	0.03	400,000	not reliable

[a] Values in these columns were either taken from the literature or calculated from recorded data.

inoculated with 1 preparation and the opposite leaf, which is equally susceptible or nearly so, can be inoculated with another preparation. A more reliable procedure still is the half-leaf method (41, 91) in which the opposite halves of the same leaves are inoculated with 2 different preparations. This is the preferred method if the leaves of the test plant are large enough, since it reduces to a minimum the variation due to inhomogeneity of the different plants and of different leaves of a single plant. Holmes (41) reports that opposite halves of a leaf are equally susceptible to infection. If this is true, and it appears to be, then variations in lesion counts where the half-leaf method is used must be due primarily to variations in the inocula or to variations in the way the different halves are rubbed.

For the most reliable results with this method one should designate a right and a left side and inoculate the right sides of half of the leaves with one preparation and the left halves of the same leaves with the second preparation. The right halves of the remaining leaves should then be inoculated with the second preparation and the left halves with the first. This will provide a balance against unequal manipulation of the 2 halves of a leaf.

Many experiments have been conducted with a view toward improving the biological assay of plant viruses. Most of these have been concerned with the production of uniform test plants or with the development of uniformity of the inoculation procedure. I shall not attempt a complete resume of this work here but shall limit the presentation of this material to a list of some of the environmental factors that have been shown to have an influence upon the production of local lesions in an inoculated host plant. The use of fine carborundum, of diatomaceous earth, of aluminum oxide, or of some other fine abrasive during inoculation has been shown to increase the number of local lesions or of systemic infections that can be obtained (44, 85, 86). Reproducibility has been demonstrated to be affected also by temperature (45, 67), light (7, 66, 67, 114), physiological condition of the plants (92, 126), age of host (91, 126), host nutrition (5, 87), inhibitory substances in the host, (29, 69), inhibitory or helpful substances in the inoculum (29, 41, 124, 125), plant species or varieties (75, 124), genetical differences among plants of a species (91), and in the method of inoculation and the quantity of virus employed (70, 90, 91, 99, 128).

In this sort of assay of plant viruses, the preparations that give the most reliable results contain from 10 million to 100 billion characteristic particles per ml. For TMV the best concentration appears to be around 10 million particles per ml. This amounts to approxi-

mately 0.000001 mg/ml and, since one needs about 5 ml for an adequate assay, a minimum of about 0.000005 mg or 50 million characteristic particles are needed for an adequate assay by the local-lesion method (Table 1).

Since adequate reviews of the statistical analyses of the local-lesion method of assaying plant viruses are already available (4, 52, 79), I shall report here only the information relative to the reproducibility of the results. With tobacco mosaic virus, Spencer and Price (99) were able to determine the activity of an unknown with an error not exceeding 10 per cent. To do this, they conducted a preliminary experiment to determine the approximate activity of the unknown. They then prepared 2 dilutions of the unknown of which the more concentrated would produce from 15 to 35 lesions per half-leaf, and 2 corresponding dilutions of a standard preparation. These dilutions of the standard and of the unknown were then paired on opposite halves of primary leaves of bean plants. More recent comparisons of the same nature have been obtained with other viruses (77, 80) in which the errors were found to be 11 per cent for tobacco necrosis virus, 14 per cent for tobacco ringspot virus, 18 per cent for alfalfa mosaic virus, and 10–15 per cent for southern bean mosaic virus.

3) *Insect transmission.*—Three procedures have been employed for the assay of plant viruses by use of their insect vectors. One assay method is based upon the percentage of insects that become viruliferous after feeding for a very short period of time on certain host plants or upon a certain part of a host plant. This procedure has been applied by Sylvester (113) to the study of the concentration of brassica virus in various parts of the plant. A second procedure that has been used primarily for studies with curly top virus of sugar beets is based upon the ability of the insect vector, a leafhopper (*Circulifer tenellus* Baker), to feed through a membrane and obtain the virus agent from the food suspension (11, 12, 22, 23, 24, 35, 96). Different batches of these leafhoppers are permitted to feed upon different dilutions of suspensions of curly top virus. The relative virus content of 2 or more samples can be roughly estimated from the number of insects that become viruliferous after feeding on the different dilutions of each. The third procedure has been applied to studies with other leafhopper-transmitted viruses and involves the injection of various dilutions of virus suspensions into the abdomen of the leafhopper vector (13, 14, 62, 63, 112). Here the relative concentration of the virus in different preparations is estimated from the number of insects that become viruliferous following injection.

Truly quantitative studies are yet to be made with the insect transmission of plant viruses but the recent work of Black and Brakke (15), Black and Lee (16), Brakke (18, 19, 20), and Brakke, Vatter, and Black (21) encourages the hope that such studies soon will be possible. When it is possible to purify and to count the characteristic particles for these viruses, it should be possible to inject known numbers of particles into leafhoppers and to estimate the number needed to produce viruliferous insects.

At present, one can say that the only distinct advantage of the insect-transmission methods is that, crude though they are, they are the only procedures by which the relative infectivities of many of the plant viruses can be determined. The disadvantages, however, are many. One has the problems of susceptibility and resistance of the plant host that are encountered with the assay of mechanically-inoculable plant viruses plus similar variabilities among the insect vectors.

Even though insect transmission of TMV has been reported by Hoggan (38), Walters (117), and others, no effective use of an insect vector in assaying this virus has been developed. Consequently insect transmission is not included in Table 1.

4) *Grafting.*—Plant viruses that can be transmitted mechanically or by insect vectors are also capable of being transmitted through graft unions. Other viruses have been transmitted through such unions only. Any of the standard grafting procedures usually are sufficient for the transfer of virus from an infected scion to a healthy stock. The use of various species of dodder as graft links between infected and healthy plants has proved useful for the transfer of viruses from plants of one family to plants of a different family where normal graft unions will not develop. Although grafting permits the passage of virus from one host to another and is commonly employed to test nursery stocks for the presence of virus, no adequate methods have been developed yet for the assay of virus quantities by a procedure involving grafts.

Other procedures for the assay of plant viruses.—We now turn to the nonbiological methods by which it is possible to determine the content of characteristic particle material in a given virus suspension. Many of these methods are important as supplements to the biological procedures, and to each other, even though they cannot be used to assay the infectivity of a preparation. When employing any of these nonbiological assay methods, one must not forget that he is measuring quantity, volume, number of particles, or some other property rather than biological activity. We should consider each method a useful tool and should try to discern its advantages and disadvantages. It is often true that one would prefer to use a given procedure but that the equipment or help is not available. Under such circumstances a substituted procedure which may not be the best one for the given study may be satisfactorily employed.

1) *Serological procedures.*—Following the early work of Beale (8, 9, 10) on the serology of plant viruses, many procedures have been used for the application of serological methods to the assay of plant-virus preparations. Several methods have proved effective and should be discussed here.

a) *Precipitin reaction.*—Quantitative investigations of the precipitin reaction between TMV and its homologous antibody are reviewed by Malkiel and Stanley

(61) who report further investigations of this nature. They found that increasing amounts of antigen added to a constant volume of immune rabbit serum produced increasing amounts of precipitate with a maximum in the region of antigen excess. For a representative experiment they needed approximately 4.5 mg of TMV for a single examination or 9.0 mg if duplicate samples were run. In a later study, Malkiel (60) compared the quantitative precipitin reaction of chemically-treated TMV samples with that of native TMV. For these studies he used only 5 dilutions of each antigen suspension. On the assumption that he could make accurate determinations with 0.03 mg of precipitate nitrogen as reported in the earlier paper (61) and noting that duplicate precipitates were obtained, it is assumed that approximately 1.8 mg of each antigen were used for the comparison. This appears to be the lower limit of the amount of antigen generally used where the quantitative precipitin reaction is employed. From the results shown, however, it appears that one could gain a relatively accurate estimate of the quantity of TMV antigen present in a sample by obtaining duplicate precipitates of a single dilution of the unknown containing approximately 0.18 mg of virus, if the serum used has been properly calibrated against a standard.

A different application of the precipitin tests has been employed by Matsumoto (64, 65) to determine the relative quantities of virus in various parts of a plant. This procedure involves the use of capillary tubes into which 0.05 ml of antiserum diluted 2-fold is pipetted. An equal volume of clarified, diluted infectious juice is then layered on top of the antiserum and the tubes are incubated at 37°C for 30–60 minutes. Determinations are made by "reading the presence or absence of flocculence or precipitate at the zone of contact and also the grade of this flocculence." From the quantities of material used in these experiments and from the quantity of virus per gram of green weight found by Steere (110) for inoculated leaves, it can be estimated that this procedure is sufficiently sensitive to permit reliable assays on 0.05 ml of suspensions containing 0.1 mg/ml. Thus a total of 0.005 mg of virus is sufficient for an assay. If this procedure were applied to the assay of purified TMV suspensions, even smaller samples might be sufficient for an assay.

The advantages of the precipitin test are that it is rapid and easy to use to detect the presence of a given or related antigen in a virus suspension and that it can be used under certain circumstances as a quantitative procedure for determining the amount of antigen present in nonpurified virus suspensions as well as those which are highly purified.

b) *Modified precipitin test.*—A new serological test has been reported recently by Moorhead and Price (74) in which red blood cells of sheep are employed for the detection of the antigen-antibody combination. In this test 2-fold serial dilutions of antiserum and of the virus suspension were prepared with buffered saline. One-tenth ml of each dilution of antigen and of antiserum were transferred to wells in a plate in such a manner that each dilution of virus was mixed with each dilution of antiserum and with adequate controls containing serum alone and antigen alone. Two-tenths ml of a freshly prepared 1 per cent suspension of washed sheep red blood cells in buffered saline was then added to each mixture and the solutions were thoroughly mixed and placed at 5°C for 3 hours. At the end of this time, the pattern of settled cells revealed the extent of antigen-antibody combination.

No reference has been located reporting the use of this procedure for the assay of plant viruses, but Moorhead and Price (74) do suggest a way to use this test as a relatively simple method for determining the concentration of TMV in purified preparations. In the same paper, Moorhead and Price reported that this procedure gives approximately the same results as those obtained with the quantitative precipitin reaction. This modified precipitin test, however, involves the use of only 0.1 ml of antigen and antiserum, whereas 0.5 ml of each is used for the quantitative precipitin test. They also report that this test is more sensitive than the precipitin reaction in that the latter tests gave trace reactions in a large number of higher dilutions of both antigen and antiserum, making detection of a precise end-point more difficult than in the modified precipitin test. With this test it should be possible to obtain reliable assays with 2.2 mg of purified TMV. Since only 0.1 ml is needed for each precipitation, approximately 1.2 ml are sufficient for an assay of a sample for which a good estimate of the concentration can be made in advance. An original concentration of virus at 1.8–2 mg/ml can be used for this sort of assay. To run a complete assay as outlined in the paper, a total of 13.8 mg of antigen is required.

c) *Complement fixation.*—The complement fixation test is based upon the fixation of complement, a substance that produces lysis of blood corpuscles when mixed with them in the presence of a third substance called the haemolytic ambocepter. When the complement is present during the reaction between antigen and antibodies it is "fixed" and becomes incapable of inducing this lysis of the blood corpuscles. The haemolytic ambocepter used in these tests consists of serum from rabbits that have been immunized by injection of sheep-blood corpuscles. The thermolabile complement which is present in the haemolytic amboceptor is destroyed by heating to 56°C. Standardized complement is provided by guinea pig serum and washed sheep-blood corpuscles are used. In practice, the complement, antigen, and antibodies are mixed and held for from an hour to a day at 4°C for the antigen-antibody reaction to take place. The red blood cells and haemolytic ambocepter are then added and the preparation is incubated at 37°C for 30 minutes. Readings are made in terms of the degree of lysis of the sheep-blood corpuscles.

This procedure, which has been used by Weaver and Price (118) to differentiate strains of TMV, has the

disadvantage of being more complicated and requiring more components than does the precipitin test, but it can be used to advantage for certain studies because it is a more sensitive test of the antigen-antibody reaction than is the precipitin test. Moorhead and Price obtained an optimum antigenic unit of 0.76 mg/ml for the precipitin test and for the red-blood-cell precipitin test. With the same sample of material the optimum antigenic unit when employing the complement fixation procedure is 0.0059 mg/ml. The minimum dry weight of characteristic particles of TMV which gives a reading with the complement fixation procedure is about 0.0007 mg or 1/10 as much as the minimum required for the ring-test precipitin reaction.

2) *Particle-count method.*—In 1950, Backus and Williams (3) described a method for quantitative assay of the number of characteristic particles in purified virus preparations. The procedure requires the use of a suspension of particles of uniform size and of known concentration as a standard. In this work, Dow polystyrene latex, lot 580 G (1, 37), is commonly used. The latex suspension and virus suspension are mixed in known proportions and are sprayed onto collodion-coated grids prepared for the electron microscope. A spray gun (3) or atomizer (119) is used which produces droplets of a size such that many of the residues of dried droplets may be photographed in their entirety with the electron microscope. Ten or 20 entire drop residues are photographed and the number of latex particles and the number of characteristic particles for the virus being studied are counted. From these counts, the number of characteristic particles per ml can be determined. The procedure has an advantage over most other nonbiological assay methods in that relatively impure preparations from infected plants can be examined; all that is required is that the characteristic particles be identifiable in the micrographs (110). This not only decreases the amount of work required for a single assay but it also prevents much of the loss of virus that is unavoidable where purified preparations are required. This is one of the most sensitive assay methods (2), since only 10 million particles are required for the entire assay (Table 1). This procedure has been reported to be reproducible to within 5 per cent (3).

The particle-count procedure has 2 distinct disadvantages. It requires a concentration of around 1 billion particles per ml (although only a fraction of a ml need be used) and, like all nonbiological procedures, it cannot be used to distinguish between infectious and noninfectious particles. In theory, one should be able to use an adaptation of this procedure to count the number of particles present in highly purified suspensions of much lower particle concentration. If one could evaporate 1 ml containing only 1 particle on a very small area of a collodion film and know that the particle had not been lost, it should be possible to photograph that particle. This is the extreme case, but it certainly should be possible to make adequate assays of purified characteristic particles where the concentration in particles is well below that now necessary. In fact, a procedure has been described (97) which permits the adequate determination of particle numbers in suspensions 1000-fold less concentrated than that needed for the spray technique. This procedure involves sedimentation of the characteristic particles from suspensions containing as few as 1 million particles per ml onto an agar surface with subsequent removal in a collodion pseudoreplica. As far as is known, this procedure has not yet been used to determine the number of characteristic particles in a preparation of plant-virus material.

3) *Dry weight of purified virus.*—One of the most obvious procedures and consequently one of the earliest nonbiological procedures used for the assay of plant viruses is that of obtaining the dry weight of purified preparations of the characteristic particles. If one is to assume that such an assay is truly an assay of the virus content of the sample, one must previously make the assumption or obtain substantial evidence that there is an infectious unit of a specific size and that the undried preparation consists of a suspension of that kind of unit only.

Much of the early work with purified suspensions of plant viruses was based upon the values obtained by weighing dried samples of purified material. Quartz-fiber balances now are available that are capable of weighing rapidly and reliably to $\pm$ 0.000005 mg (46). This means that one can weigh a 0.0001 mg sample with an error no greater than 5 per cent. This procedure gives the weight of all the nonvolatile material present in the dried sample and has the distinct disadvantage of being incapable of detecting impurities. It is important, therefore, that samples to be assayed by this procedure be examined by all the known methods that can detect impurities and that no recognizable impurities be present.

4) *Chemical assays.*—Provided pure suspensions of characteristic particles can be obtained, it is possible to make rapid and reliable assays of the quantity in a suspension by microchemical procedures. The quantity of some constituent of the particles, usually nitrogen or phosphorous, is determined for a standard weighed sample of purified material. Unknown purified virus suspensions then can be reliably assayed by determining the amount of one or more of these constituents in very small samples. Present microchemical procedures permit the assay of samples containing as little as 0.0005 mg of nitrogen or phosphorous with an error of less than 5 per cent (46). This amounts to about 0.003 mg of the purified nucleoprotein or 30 billion characteristic particles for a TMV suspension on the basis of nitrogen determination (Table 1) or 0.010 mg (100 billion characteristic particles) on the basis of phosphorus determination . When one conducts an assay by chemical analysis one is dependent upon the purity of his preparations. Any nonvirus component in the suspension can cause errors in the determination. Also one must be careful not to introduce, during preparation, buffering salts of any con-

stituent on which the analysis is to be based.

5) *Optical density.*—Again a purified sample must be dried and weighed. This sample of known concentration must be diluted and a family of curves of the optical density at different wave lengths for different concentrations must be obtained as a standard. As employed (94, 115), this procedure has been used for TMV assays. Readings were made at a single wave length, 2600 A. For other viruses, absorption at a different wave length conceivably might give more reliable assays. After this family of curves has been obtained it is relatively easy to assay purified virus suspensions of unknown concentration by taking the necessary readings and comparing them with the corresponding curve of the standard preparation. Samples containing approximately 0.02 mg of purified material or 300 billion particles can be assayed by this procedure with an error of less than 5 per cent (Table 1).

The advantage of this method as compared with chemical assays is that none of the suspension need be used up in the assay procedure although inactivation of infectious material may occur. This procedure also has the distinct advantage of enabling one to make rapid assays of highly purified material. It is important that the preparation be highly purified or at least freed of any contaminating component that will absorb at the wave length used. Extraneous components have been found to interfere with the reliability of this assay procedure for measuring the quantity of material in TMV preparations in some samples prepared by a rapid purification procedure (94).

6) *Colorimetric procedure.*—Lojkin and Beale (57) have suggested the use of a colorimetric procedure for the quantitative determination of purified suspensions of TMV characteristic particles. They report that such quantitative determinations can be obtained with purified preparations containing 0.008 to 0.6 mg of material. In this procedure, the Folin phenol reagent (34) is added to the suspension and mixed thoroughly. Readings are then obtained with a photoelectric colorimeter. Commoner and coworkers (26) report a similar assay procedure with essentially the same limitations.

7) *Critical dilution of stream double refraction.*—This procedure depends upon the size and the rod-like shape of the characteristic particles in TMV preparations. Numerous dilutions of a virus preparation are prepared and examined for the presence or absence of stream double refraction under a polarizing microscope. The dilution point at which stream double refraction first fails to occur is recorded. Takahashi and Rawlins (116) suggested that this procedure might be used as a rapid quantitative assay method for TMV. Lauffer and Stanley (53), however, found variability in the critical dilution point of different samples which depended upon the preparative technique used rather than upon the infectivity or upon the amount of purified protein present. They observed that changes in pH, which are known to have an effect upon the degree of aggregation or disaggregation of the characteristic particles, had a marked effect upon stream double refraction. These findings have limited the use of this procedure for virus assays. It is unfortunate that this variability in results occurs. Otherwise the procedure could be used as a rapid test of the concentration of rod-like particles in rather impure preparations containing as little as 0.03 mg of such material (53), Table 1.

Combined use of 2 or more procedures.—Now that we have discussed each of the assay procedures individually it might be well to compare them, and to elaborate on the use of various combinations of 2 or more methods to obtain added information. A quick reference to Table 1 is sufficient to impress one with the fact that 2 of the assay methods are considerably more sensitive than the others. Only 0.000001 mg (dry wt.) per ml of a purified suspension of the characteristic particles is necessary for local-lesion assays with TMV. For adequate comparisons at 2 concentrations, about 5 ml of this concentration are necessary. Thus, a complete biological assay can be made with a suspension containing approximately 50 million characteristic particles. For the electron-microscope particle-count procedure a concentration of one billion characteristic particles per ml is needed but only 0.01 ml is required for an assay, so the total number of characteristic particles necessary for such an assay is only 10 million. These weight calculations are based on the weight of the characteristic particle of TMV as determined by Williams, Backus and Steere (122). Use of the particle-count procedure in combination with a local-lesion procedure has made it possible to demonstrate that 1 lesion can be obtained for every 50,000 characteristic particles in a fresh inoculum of TMV (111). To my knowledge this is the highest ratio of infectivity to number of characteristic particles yet obtained for a plant virus. This value was obtained with juice from leaves of young Turkish tobacco plants that had been infected for 2 weeks and that showed characteristic symptoms of TMV. The juice was diluted with an equal volume of distilled water, heated for 10 minutes at 60°C and clarified by centrifugation for 15 minutes at 3,000 rpm in an International clinical centrifuge. Samples of the amber supernatant liquid were diluted for particle counts according to the procedure of Backus and Williams (3). When the counts were completed and the concentration of characteristic particles in the suspension was calculated, other samples were diluted for inoculation. This time, however, an inoculation procedure distinct from that normally used was employed. A ½-ml sample containing 32 million particles per ml was measured into the middle of a 4-in. watch glass. Fine 600-mesh carborundum was added to the ½-ml sample. Inoculation was made by the dipping of an index finger into the mixture and the transferring of very small quantities to the leaves of local-lesion host plants. These small samples were rubbed over the leaf surface gently but firmly until the

entire ½-ml sample was spread over as many leaves as possible. The center of the watch glass was washed with a drop of buffer which was then used to inoculate still more leaves. Every lesion that appeared was counted. The sample used contained 16 million characteristic particles, and a total of 305 lesions were obtained. This amounts to 1 lesion per 52,460 characteristic particles or, in round numbers, 50,000. The plants used for this experiment were not optimally receptive and it is believed that, with more uniformly receptive local-lesion host plants and with greater care to make use of all the inoculum and to employ only the most receptive areas of the best local-lesion hosts, it will be possible to obtain many more lesions with a preparation containing this number of particles. This procedure has also been applied to a preparation of tobacco ringspot virus for which 1 lesion was obtained per 120,000 particles.

Two of the procedures discussed here have been used together to determine the weights of the characteristic particles for tomato bushy stunt virus (121) and TMV (122). For this purpose the number of particles per ml was determined by the particle-count procedure and a measured volume of a more concentrated suspension was dried and weighed. From the information obtained by use of these 2 procedures it is possible to determine the weight of a characteristic particle. The particle weight as determined by this procedure has been used to determine values given in Table 1.

COMPARISON OF PLANT-VIRUS ASSAYS WITH ANIMAL- AND BACTERIAL-VIRUS ASSAYS.—If we are able to observe only 1 infection for every 50,000 virus particles present in an inoculum it is obvious that only a small fraction of the particles are infectious or that the inoculation procedure is very inefficient. One is inclined to ask, therefore, how plant-virus assays compare with animal-virus and bacterial-virus (bacteriophage) assays. The association of infectivity with characteristic particles is found to be far better for the bacteriophages and for several of the animal viruses than for any of the plant viruses. In 1939 Smadel, Rivers and Pickels (98) obtained 1 lesion for every 4.2 particles with vaccinia virus although their results involved the assumption that their preparations were pure and that the weight of a single particle was known. Luria, Williams and Backus (59) by using the particle-count technique were able to show with T2, T4, and T6 phages that essentially every particle was able to initiate an infection. More recently, Crocker (27) has obtained a lesion for every 43 elementary bodies with the virus of meningo-pneumonitis; Donald and Isaacs (28) and Levine, Puck, and Sagik (56) have obtained an infection for every 10 or fewer particles with influenza virus; and Schwerdt and McClain (95) have obtained a plaque for every 500 to 2000 particles of poliomyelitis virus. The electron-microscope particle-count procedure (3) was used in conjunction with infectivity assays to obtain these values. The polio virus was assayed for infectivity by means of the plaque technique of Dulbecco (31, 32). Infectivity titrations of preparations of the other 2 viruses were carried out with eggs.

It is still true that when poliomyelitis virus is inoculated into animals, or when any of many other animal viruses is inoculated into animals or into eggs, the greatest dilutions at which 50 per cent of the inoculations result in infection contain 100,000–1,000,000 or more characteristic particles per infection. Only the recent application of plaque techniques to such viruses as those of poliomyelitis and equine encephalitis has made it possible to obtain the increased numbers of infections for a given concentration of particles of these viruses.

SUGGESTIONS FOR IMPROVEMENT OF PLANT-VIRUS ASSAYS.—We have considered various concepts concerning the assay of plant viruses. The major inherent problem, that of determining the number of infectious units in a sample, has been discussed, but little attention has been paid to its ultimate solution. We have observed that, with some animal and bacterial viruses, infection can be induced by fewer than 50, and in some instances by essentially every characteristic particle in a purified preparation. This observation, and the very definite association of infectivity of plant viruses with characteristic particles, encourages the hope that some day it may be possible to show conclusively that nearly all characteristic particles of fresh preparations of the more stable plant viruses are able to initiate infection. It seems appropriate, therefore, to look to the future and to contemplate experiments that may lead to a more precise determination of the total number of infectious units present in a sample. It is quite probable that the method described by which 1 lesion has been obtained for every 50,000 characteristic particles can be improved to a point where 1 lesion can be obtained for every 5,000 particles or even for every 500 particles, but it is very doubtful that 1 lesion ever can be obtained for every 100 or fewer characteristic particles. Improvements can certainly be made in the selection of the most sensitive local-lesion hosts and in the environmental conditions under which the test plants are propagated. Also, much more skill can be developed in the spreading of the inoculum over more leaf area and only over the most sensitive areas. It should be possible to wash the inoculated leaves and recover the virus that remains on the surface, to concentrate the recovered virus by centrifugation, and to inoculate additional leaves with the recovered virus. Such recovery could be continued to a point where additional washing and concentration of the recovered virus fail to produce additional lesions. This procedure is the most obvious way to reduce rapidly the gap that now exists between number of infections obtainable and total number of characteristic particles in an inoculum. We must not depend upon it, however, for the ultimate association of infection with fewer than 100 particles, but must turn elsewhere for methods by which this may be made possible.

One alternate procedure which has been attempted with some success (88) is the microinjection of virus into single cells. It is hoped that future experiments of this nature might be even more successful and that ideal conditions can be imposed for the production of a lesion for every injected cell that receives an infectious unit. It is highly probable that the buffering medium, the size of the needle, and the volume of inoculum injected will have an important influence upon the results obtained.

Another procedure that conceivably could be a solution to this problem is the use of a plaque technique similar to that developed by Dulbecco (31) for animal-virus assays. A suspension, in the proper buffer, of parenchyma cells of a local-lesion host might expose a sufficient area of cell membrane through the openings in the cell walls formerly occupied by plasmodesmata to permit most of the infectious units to initiate infection and to produce plaques of chlorotic or of necrotic cells. The parenchyma cells might be produced in tissue cultures or might be obtained from homogenates of leaves or stems of growing plants. A slight variation of this method might be the use of the inside surface of freshly prepared epidermal strips, or of the layer of parenchyma cells exposed when the epidermis is removed. Such tissues might well be subject to infection without further mechanical injury, especially if an ideal buffering medium is used, and if the stripping is done in the presence of active virus so that the virus can make good contact with the naked protoplasm while the plasmodesmata are being broken. The advantage of using tissue or cell suspensions from local-lesion hosts for such studies is that plaques or local lesions could be distinguished easily without the necessity of developing vital stains for the detection of virus-infected cells, as might be required if cells of systemic host plants were used.

The purification of some of the leafhopper-transmitted viruses and the improvement of procedures for injection of microquantities of virus suspensions into nonviruliferous leafhoppers lead one to hope that before long it may be possible to make counts of the number of characteristic particles of some of these viruses in purified samples and to inject small volumes containing very few such particles into each of a large number of nonviruliferous leafhoppers. It might be possible with such a procedure to obtain infection with samples known to contain as few as 10 or 20 particles. Such information certainly would be welcome but might not shed much light on the number of infectious particles present in samples of the common mechanically-transmitted viruses.

In conclusion it might be well to emphasize the fact that the total number of characteristic particles present in a purified virus suspension can be rapidly and reliably determined by the electron-microscope particle-count procedure. Other procedures that tend to be more critically influenced by the presence of contaminants of very small size relative to the characteristic particles are commonly used to advantage in the assay of purified suspensions. Serological procedures are used on both purified and nonpurified suspensions to test for the presence of or quantity of antigen in a sample. We also have very adequate procedures for comparing the relative infectivities of 2 or more samples. What we do not have and really need is positive evidence that a single characteristic particle and an infectious unit are one and the same. This is our major problem in the assay of plant viruses and deserves considerable attention.

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CONCEPTS AND PROBLEMS CONCERNING PURIFICATION OF LABILE INSECT-TRANSMITTED PLANT VIRUSES[1]

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In this paper an attempt will be made to picture some recent progress accomplished with the unstable insect-transmitted plant viruses, not only by describing recent results but also by attempting an evaluation of their significance, examining some of the questions raised, and hazarding hypotheses on their solution. In doing so, pertinent allusions will be made, principally to experiments with potato yellow-dwarf virus, *Aureogenus vastans* (Holmes) Black, wound-tumor virus, *Aureogenus magnivena* Black and tomato spotted-wilt virus, *Lethum australiense* Holmes, 3 viruses with which I am familiar.

The purification that will be discussed is largely a matter of the separation of evanescent virus particles from other colloidal particles occurring in extracts of host cells and the identification of the presumed virus particles, with specific infectivities, visualized under the electron microscope. We are just reaching the stage in purification of these particular viruses where we can begin to concern ourselves with problems requiring that these viruses be free of extraneous chemical substances. Purification to this degree and the knowledge it permits must be a subject for the future.

The electron microscope has provided the virologist with one of the most important instruments that can be applied to viruses. Electron micrographs are rapidly providing us with better information about the size and shape of viruses than could be obtained by techniques of filtration and physical chemistry. One of the valuable features of the electron microscope is that it requires a drop of solution no larger than about 0.5 μl to prepare a single mount. However, it is our experience that the virus to be examined in this drop should be quite concentrated in relation to concentrations to which we are accustomed when determining infectivity. These attributes make it possible and sometimes advantageous to work with small volumes of starting material of the highest virus concentration in attempts to identify labile plant viruses under the electron microscope. Experience indicates that low initial virus concentration in the host, in addition to instability, is one of the obstacles to be overcome if success is to be attained with some of these viruses. Consequently the difference between success and failure may be determined by the use of one source of virus rather than another at the very start of the experimental procedure of isolation. It is, therefore, important to consider a number of factors well known to affect virus concentration in the host.

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THE SOURCE OF VIRUS.—There may be important differences in concentrations attained by different strains of a virus. Stanley (41) showed that the common tobacco mosaic virus, *Marmor tabaci* Holmes, yielded twice as much isolated virus as did Holmes' masked strain, *M. tabaci* var. *obscurum* Holmes, when grown in the same host. Before beginning our work on tomato spotted-wilt, 5 collections of the virus from different parts of the United States were studied and it was found that one of them collected by Dr. M. W. Gardner in California was regularly more infective than others. This collection was chosen for attempted identification under the electron microscope (8). Until progress in purification has been achieved, virus concentrations usually must be inferred from relative infectivities or possibly from serological titers, although neither may be correlated exactly with relative virus concentrations.

The host plant selected as a source of virus is also important. Stanley (41) found that spinach, *Spinacia oleracea* L., yielded less than one tenth the amount of isolated tobacco mosaic virus that was obtained from the same weight of tobacco, *Nicotiana tabacum* L. In some cases in our work the choice of host was obvious. *N. rustica* L. is far superior to other known hosts as a source of potato yellow-dwarf virus and has been employed consistently in purification studies. In other instances the choice was, at first, not so apparent. In our work with wound-tumor virus, *Rumex acetosa* L. was tested as a source because of the striking symptoms produced. However, the clarified juice of this plant gave no test for protein and precipitated proteins very effectively, probably because of the tannin it contained. Earlier, Bawden and Kleczkowski (1) reported this same difficulty with leaves of strawberry (*Fragaria* sp.). Subsequently the very susceptible crimson clover, *Trifolium incarnatum* L., was employed as a source of wound-tumor virus for years before we decided that although the insect-injection technique was then laborious, slow and erratic, it would have to be employed to estimate infectivities of other sources. In retrospect, it is now apparent that in spite of the difficulties, we should have studied the concentration of various sources of this virus at an earlier stage in the investigation.

There is also good reason for not overlooking the variation in virus content or available virus content in extracts from different parts of the plant. For example, the various parts of cucumber plants, *Cucumis sativa* L., except the corolla, have been shown to contain an inhibitor that markedly reduces infectivity of cucumber mosaic virus, *Marmor cucumeris* Holmes, (37). Sill, Burger, Stahmann, and Walker (37, 38) demonstrated that the infectivity of cucumber corollas may be about 50-100 times that of cucumber leaves or of whole plants infected with cucumber mosaic virus. This discovery was obviously of the greatest importance in their work on the electron microscopy of the labile, insect-transmitted cucumber mosaic virus. Similarly we (14) were greatly aided in our work with wound-tumor virus by discovering that although nontumorous parts of diseased sweet clover, *Melilotus alba* Desr., contained less virus than did crimson clover, the tumors on sweet clover nevertheless contained more concentrated virus than did diseased crimson clover and roughly 100 times more than did the nontumorous portions of diseased sweet clover plants. According to our present information, the most concentrated source of this virus is the sweet clover tumor.

Consequently, we have become much interested in tumor yields. It is very evident to us now that a tumor yield from individual plants can be secured that is far superior to what we originally obtained. High tumor yields can be provided by the grafting of infected sweet clover scions of clone C10 (6) on young healthy C2 stocks and the growing of a plant that has a C10 top and a C2 root. It is not uncommon to obtain 4 g of C10 stem tumors and 11 g of C2 root tumors from such a plant and it is very probable that experimentation will provide combinations with still better tumor yields.

Nor should the investigators' attention be confined to the plant hosts. The leafhopper vector, *Agallia constricta* Van Duzee, weight for weight, is almost as good a source of wound-tumor virus as is the sweet clover tumor, and the virus has been isolated from this vector (14). Incidentally, the virus has the same form in the insect vector and in sweet clover. The aster-yellows virus, *Chlorogenus callistephi* Holmes, which has not yet been photographed and which typifies a whole group of plant viruses, appears to occur in higher concentration in the vector than in aster, *Callistephus chinensis* Nees, or in *N. rustica*. In preliminary experiments, extracts of *N. rustica* injected into the vector, *Macrosteles divisus* (Uhler), produced only a small number of infective insects. Asters were an even poorer source. Infections with virus from asters were obtained only by making extracts in solutions of Na_2SO_3 strong enough to preserve the green color of the juice, sedimenting the virus in such extracts by centrifugation, and resuspending it in a solution that could be injected into the vector without harmful effects. On the other hand, there is little difficulty in obtaining transmission from viruliferous insect juice diluted to 10^{-3} in saline. The small weight of each leafhopper, about 1 mg, is a serious handicap, but as shown with wound-tumor virus, it may not be an insurmountable one, and because of the small volumes that can be used for electron microscopy, the vector may be the best source with which to work.

The tumors caused by wound-tumor virus contain a high proportion of a phloem-like tissue, designated "pseudophloem" by Lee and Black (26), and there is a possibility that this may have much to do with the high virus content of this tumor. Many of the labile insect–transmitted viruses are phloem-specialized, and production of such overgrowths by wound-tumor virus on plants also infected with viruses like

aster-yellows might provide rich sources of the latter kind of viruses.

The rise and fall of virus concentration with the development of the acute and chronic stages of several virus diseases are well known and are well illustrated by our experience with tomato spotted-wilt in *N. rustica*. In this case, the peak in virus concentration was reached before the maximum severity of symptoms and then there was a rapid drop. The importance of harvesting material at this peak concentration is obvious. As a result of repeated experience, heavy initial inoculation has been found important in reaching the highest levels of spotted-wilt virus concentration. It is assumed that much more virus is thereby introduced into the growing point by translocation from the site of inoculation than is the case with a light inoculation. Although we may not be dealing with acute and chronic stages in wound-tumor disease, we know that here too the stage of the disease is important; vigorously growing tumors have given better virus yields than old tumors.

It need hardly be said that the growing of plants under optimum conditions for high virus concentration should not be overlooked. In early work on the purification of wound-tumor virus, negative results were not infrequently obtained from both plant tumors and from insects, apparently because of the conditions under which the source material was produced. Of the various environmental factors that are known to affect virus concentration, probably adverse temperature and light conditions are the most important sources of trouble. Kunkel's (22, 23) experiments on the destruction of aster-yellows virus in both plant hosts and insect vectors at temperatures above 30°C indicate that in attempts to purify aster-yellows virus one may expect that growing the virus in insect or plant at favorable temperatures will be of critical importance. In cooperative work with Dr. Frits Went we have been interested in determining the temperature and light conditions that provide a maximum yield of tumors incited by wound-tumor virus. A 16-hr. light period at 20°C and an 8-hr. dark period at 17°C was one of the most favorable environments for this purpose and plants grown under such conditions yielded a considerably greater weight of tumors than did plants grown in a number of other environments (45). Tumors grown under these conditions were later found to yield virus consistently and in sufficient amounts for isolation.

The factors mentioned do not exhaust those known to affect the virus concentration in the host cell. There have been many studies on the effect of nutrition on virus concentration following Spencer's (39) report that tobacco mosaic virus was 80 times more concentrated in juice from plants supplied ample nitrogen than it was in juice from plants starved for nitrogen. Ross, Rochow, and Siegel's (36) report that the concentration of potato X-virus, *Annulus dubius* Holmes, in leaves also infected with the unrelated potato Y-virus, *Marmor upsilon* Holmes, was 10 times greater than in leaves infected with X-virus alone indicates some unusual influences that may affect virus concentration.

METHODS OF PURIFICATION.—The first virus to be isolated, tobacco mosaic virus, was purified initially by chemical methods (40). Later, less stable viruses were purified by the physical method of cycles of alternating high- and low-speed centrifugations at low temperatures (42) and since then purification by alternating high- and low-speed centrifugations in appropriate solvents and preferably at temperatures near freezing has become a standard procedure. The first of the highly labile insect-transmitted viruses to be detected under the electron microscope, potato yellow-dwarf virus, was observed in extracts prepared by this technique (9). However, much better preparations of this virus have been obtained, and other labile viruses isolated, since Brakke invented a density-gradient centrifugation technique (10) and demonstrated its value in isolating these viruses. More recently he has modified zone electrophoresis (12) so that it may be carried out in a density-gradient column, and this has permitted still further purification.

Brakke's techniques were developed during persistent efforts to identify the wound-tumor virus under the electron microscope and after consistent failure to make progress by current methods such as chemical precipitation, cycles of high- and low-speed angle centrifugation at low temperatures, electrophoresis in the Tiselius apparatus, adsorption and elution on chromatographic columns, adsorption on chicken red blood cells, examination of extracts by spectrophotometry, and examination of sectioned tissue under the electron microscope. The continued support of the American Cancer Society through this long period of failure is much appreciated. After many failures with the wound-tumor virus it seemed advisable to concentrate on attempts to improve the effectiveness of centrifugation and to use potato yellow-dwarf virus as a test virus because it already had been detected under the electron microscope and because a better infectivity assay existed for it than for any other leafhopper-borne virus.

When Brakke (10) conceived the idea of centrifuging a layer of potato yellow-dwarf virus suspension on top of a sucrose solution increasing in concentration from top to bottom of the tube, an important advance was made. Earlier work with density-gradient columns had employed different methods. Harvey (20) suggested the use of density-gradient columns during centrifugation of cells in order to suspend them in a stratum of equal density "perfectly cushioned against crushing" by forces thousands of times gravity. Linderstrøm-Lang (27) and Behrens (3) used density gradient columns to determine the densities of materials insoluble in the solutions of the column. Density-gradient columns have been used frequently since then for density determinations or for separations based on densities and in such work centrifugation often has been used to speed the attainment of equilibrium.

Brakke, on the other hand, prepared density-gradient columns of sucrose solutions, in which the virus particles were soluble, and layered the suspension to be fractionated on top of the gradient column. On subsequent centrifugation of such a column, particles will, at first, tend to separate into zones at different depths in the tubes depending largely on their sedimentation rates through water, that is, depending on size, shape and density of the particles. On prolonged sedimentation the particles will tend to approach a quasi-equilibrium where they are separated into zones according to their densities, so that the density of the particles in each zone approximates closely that in the adjacent solution. Zones obtained by either "rate" or "equilibrium" centrifugation are stable for several days if left undisturbed. The gradient columns themselves will maintain a gradient for months if kept at a uniform temperature, although the steepness of the gradient slowly decreases.

Let us imagine a very small particle of high density and a much larger particle of a density not much greater than that of water. One easily can conceive a top layer consisting of a mixture of 2 such kinds of particles which on centrifugation over a density-gradient column would at first separate with small particles of high density as an upper zone and large particles of low density as a lower zone but which on further centrifugation would separate with the positions of the zones reversed as the particles approached a density equilibrium with the adjacent solution. Thus this technique can be managed to provide 2 different fractionations. Brakke finds, in practice, that large mitochondria of low density usually are found in the virus zone after a zonal centrifugation of the sedimentation-rate type. These mitochondria are separated readily by centrifuging the virus to a position approximating equilibrium in the gradient column. Chloroplast fragments, on the other hand, are not separated from particles of wound-tumor, tomato spotted-wilt or potato yellow-dwarf viruses by equilibrium centrifugation but are separated readily by rate-zonal centrifugation.

Brakke (12) has described the adaptation of sucrose density-gradient columns to zone electrophoresis and points out a number of advantages of this technique over the ordinary methods of zone electrophoresis. In the first place, as a preparative technique zone electrophoresis has the same advantages over ordinary boundary electrophoresis that zonal centrifugation has over ordinary centrifugation. However, until Brakke's use of the sucrose density-gradient column in zone electrophoresis, this process was carried out in the interstices of a supporting column of some porous solid material such as wet starch to which the virus could be added and in which it was always hidden from view. At the termination of zone electrophoresis in such columns, the virus had to be eluted from the supporting solid medium and separated from it by centrifugation or other means. In the first runs, at least, one did not know where the virus was located in the column. In addition to the fact that the migrations cannot be observed readily, zone electrophoresis using porous solid supports to prevent convection has 2 other disadvantages, namely, the possibility of adsorption onto the porous solid and the electro-osmotic streaming of the buffer. These 3 disadvantages are absent when a liquid density-gradient column is used as the support to prevent convection.

In density-gradient zone electrophoresis, 2 density-gradient columns are prepared in 2 arms of a U-tube. The columns contain a suitable electrolyte and buffer in addition to the sucrose. The virus, suspended in a sucrose solution of selected density, is introduced by hypodermic needle and floated in a zone in 1 or both arms of the U-tube at a depth where the column is of the same density. When the difference in electrical potential is applied to the 2 ends of the U-tube, separation of the visible virus zone from visible impurities of different electrophoretic mobilities may be observed with the unaided eye. The zones of mitochondria become broader as they migrate, probably reflecting heterogeneity of charge whereas the virus zone maintains a uniform size during the migration. Chloroplast fragments may maintain a zone of uniform size but they are readily differentiated from the virus zone by color. The identification of the virus zone should, of course, be confirmed by infectivity assay. By varying the pH, the relative mobilities between some particles can be changed so that the technique is capable of considerable variation in application to a given problem of purification. The process can be carried on at or near O°C and the equipment is not expensive as is the Tiselius apparatus.

Brakke (12) points out these features of his technique, other possible applications of it, and the method of stabilizing freshly floated zones by stirring to overcome droplet sedimentation, which nullifies the technique unless corrected and which develops upon the introduction of protein and virus zones into the gradient column. It should be pointed out here that these contributions by Brakke are in their early stages of development and that they appear to be capable of considerable refinement and to be applicable to a wide range of problems. For example, Brakke (11) has made progress in applying light-scanning techniques to the zones in density-gradient columns for analytical purposes and currently is attempting to improve such methods. In these density-gradient techniques we therefore have 3 independent physical methods of separating virus particles from other particles so that they can be obtained in a highly purified state. The methods also have the advantage of being applicable in series, one after the other, without the necessity of intervening sedimentations and resuspensions of the virus.

The density-gradient columns can be used under temperature and chemical conditions that are as ideal for the preservation of the virus outside the cell as our knowledge will permit. Highly purified sucrose is available at relatively low cost, it does not ionize, and it has been reported to have a preservative action

on some viruses (18). The density gradient columns can be prepared in solutions of suitable hydrogen-ion concentration, concentration of reducing agents, and ionic strength. In our work with tomato spotted-wilt virus all 3 conditions were important, solutions of low ionic strength being necessary to maintain this virus in a dispersed state (8). Even so, certain of the viruses, e.g. tomato spotted-wilt virus and potato yellow-dwarf virus, break down rapidly enough so that a point is reached where low molecular weight material apparently is being liberated into the preparation by break-down as rapidly as it is being removed by purification. In the case of potato yellow-dwarf virus, the addition of a hardening agent such as formaldehyde provides 1 method of meeting this difficulty, for although the formaldehyde destroys virus activity, it preserves the virus morphology and makes it possible to get better pictures of the pathogen. This particular chemical has not been effective with tomato spotted-wilt virus and the search for other hardening agents might be a fruitful field of endeavor.

Brakke (13) has found that crude juice of *N. rustica* has a protective action on potato yellow-dwarf virus. During purification these protective factors gradually are lost, leaving the virus much less stable. This has effectively prevented purification of this virus beyond a certain point. Identification of or substitution for these protective factors appears to be essential to the production of highly purified, viable potato yellow-dwarf virus. Developments along this line may have great importance for future work on these unstable plant viruses. With unstable viruses, the end-product also can be improved by the application of greater force in centrifugation so as to reduce the time for purification and consequently for break-down. The same may be true in electrophoresis up to the point where higher electric potentials cause heating detrimental to the virus or to the stability of the zones.

CORRELATION OF VIRUS ACTIVITY WITH A PARTICULAR PARTICLE.—There is an increasing number of papers reporting the detection of a virus-like particle in plants having a specific disease and the absence of the particle in corresponding healthy plants. If one were to demonstrate the presence under the microscope of a certain bacterium in large numbers in a particular kind of diseased plant and its absence in corresponding healthy plants, how many plant pathologists would feel sure of the bacterium's etiological significance in the absence of infectivity tests? Similar reports on particles of virus size obviously leave something to be desired in the way of convincing evidence that the particle in question is the virus pathogen. There is a marked contrast in the evidence required for acceptance of a particular agent as a virus now and that expected at the time Stanley (40) made the important assertion that he regarded the protein he had isolated to be tobacco mosaic virus. The fact that high molecular weight proteins (28) and virus-like particles have been reported in healthy plants should inspire caution (2, 17, 24). Furthermore, there is the possibility that a virus-like particle might be a product of the disease rather than the incitant. Nevertheless, reports of particles as virus based on their presence in diseased plants and absence in healthy plants probably will increase. It is relatively easy in some cases to obtain such results by electron microscopy and, on the other hand, a very difficult and prolonged task to relate the particle to infectivity. To date, no means of demonstrating the infectivity of some plant viruses in cell-free suspensions has been found. To insist on demonstrated virus activity for all particles suspected to be viruses before acceptance of a paper for publication would prevent the dissemination of useful information. Such reports probably are bound to be published in one journal or another. It is pertinent to mention here that the first published electron micrographs of potato yellow-dwarf virus were of this sort (9). In this case, subsequent work has confirmed the interpretation made at that time. Surely such reports, however, should be considered tentative identifications, and unproved, until better evidence relating the particle to virus activity has been secured. The biological test of infectivity still reveals the most important property of a virus and is the mainstay of practically all virus research.

After application of a separation procedure, any boundary marking a steep gradient between the presence and absence of a particle provides a means of testing that particle's association with the biological activity it is suspected of possessing. A separation ultracentrifuge cell has been used by Lauffer (25) to test the coincidence of particle and tobacco mosaic virus activity at such a boundary. Later Epstein and Lauffer (16) pointed out that there is no assurance that this technique is free from errors due to convection, and they developed a technique in which any convection during a test could be detected by simultaneously determining the sedimentation of suitable particles of known sedimentation rate. They established that 2 coincidences of activity with particle may be tested by studies of sedimentation in solvents of different density. Hartman and Lauffer (19) concerned themselves with this same problem of coincidences in electrophoresis boundaries, and in a study on the electrophoresis of southern bean mosaic virus (*Marmor laesiofaciens* Zaumeyer and Harter) in solutions of 0.02 ionic strength, determined that the electrophoretic mobility curves for characteristic particle and for infectivity at various pH values required 4 empirically derived constants for its mathematical description. Each of these 4 constants provided a test for the coincidence of particle and active principle. The variation of the isoelectric point with ionic strength provided a fifth test of coincidence.

We have not applied the same tests to the labile insect-transmitted viruses; it would be desirable to do so. However, we have applied other tests for the identity of particle and specific activity.

A simple method of centrifuging in capillary glass

tubes and assaying for infectivity at various depths in the tube was not without some success in testing the association of a particular particle with potato yellow-dwarf virus activity (15), but this method sometimes gave fallacious results (14) because of currents in the tubes.

The presence of a particle in a zone, after migration in a density-gradient column under either an electrical or centrifugal force, also provides a means of testing its association with activity. The most critical regions for testing such association will be at the upper and lower margins of the zone. The purer the zone and the more precise the physical determination of the particle distribution at the zone margins and the biological determination of the activity over the same gradients, the better the evidence for the association. Unless aggregation is complete, the upper margin of the zone in a centrifuge tube will be characterized by individual virus particles whereas the lower margin of the zone may be complicated or obscured by particles clumping together. Therefore, the upper margins of the zone in centrifuge tubes sometimes are the best regions for such determinations. Centrifugation or electrophoresis in density-gradient columns possesses an inherent advantage over conventoinal methods in that the migrations of particles present in low concentrations are not distributed by convection.

In density-gradient columns even a qualitative assay can show the migration rate of virus through the column to within about 10 per cent. Evidence relating infectivity to a particular particle by a study of zones in density-gradient tubes has been obtained for tomato spotted-wilt virus (8), potato yellow-dwarf virus (11) and wound-tumor virus (14).

It is not necessary to dwell on the application of the primary-lesion test to this problem, as in potato yellow-dwarf and tomato spotted-wilt viruses. Such techniques are well known and widely used but at present there seems to be little prospect of their general application to labile plant viruses transmitted by leafhoppers. Perhaps future research will devise some means of infecting plants with most of these viruses directly. With or without the production of primary local lesions, there would be a marked advantage in eliminating the vector as an intermediary symptomless host in infectivity tests. However, to provide a practical direct assay of a leafhopper-borne virus on plants, greater success must be secured than has been obtained so far except for the potato yellow-dwarf viruses. With both curly-top, *Ruga verrucosans* Carsner and Bennett, and wound-tumor viruses, pin pricks through virus solutions into plant parts rich in phloem give very poor results. On the other hand, the insect-injection technique appears to have promise of wide utility for the leafhopper-borne viruses having long incubation periods in the vector. Storey (43) first described the successful transmission of corn-streak virus, *Fractilinea maidis* (Holmes) McKinney, by this method and it was later successfully used to transmit aster-yellows virus (4), wound-tumor virus (35) and corn-stunt virus (32). Just as Holmes' (21) primary-lesion technique provided an important means for following tobacco mosaic virus in isolation experiments, so has the insect-injection technique provided a means for detecting the presence of some leafhopper-borne viruses in cell-free extracts and hence a means of following them in fractionation procedures.

There are some aspects of the insect-injection technique which might be dealt with profitably here and there are many details that are important to the successful and efficient use of the technique that cannot be more than indicated. These include the selection of insects of suitable age for injection, the use of immune plants growing in soil free of weeds during the incubation period, the importance of temperature during incubation and transmission, and the avoidance of vectorless strains of virus.

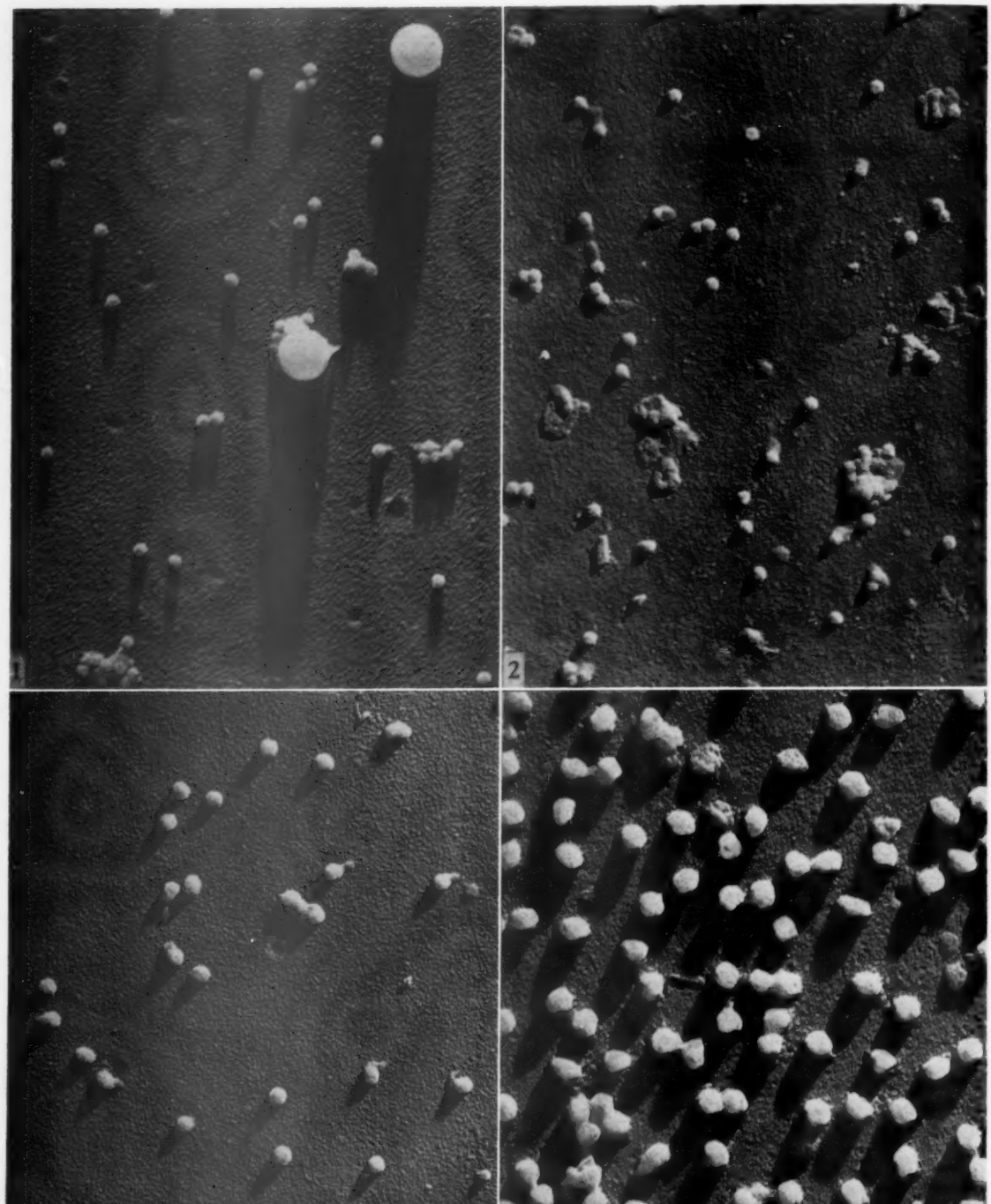
One of the remarkable features of this technique is the ability of many leafhopper vectors to withstand the injury inflicted when the virus is injected. They can survive injections in the head, thorax, or abdomen. In our experience, injection in the abdomen has been the most easily performed and has given the best survival and good transmission. In our experiments, 3 weeks after the operation the survival of injected leafhoppers is usually 40–80 per cent that of the noninjected leafhoppers.

We have regularly inoculated insects in a cold room maintained at about 0°C. This has served to anesthetize the insects and preserve the virus. It has been known for some years that even with the very unstable aster-yellows virus, insect injection could be carried out successfully at room temperature by using ether to anesthetize the insects and by keeping the virus solution in an ice-water bath. Maramorosch (33) recently has applied the CO_2 method for anesthetizing insects to the injection of leafhoppers with aster-yellows virus. This promises to be an important innovation because it eliminates the cumbersomeness of carrying out a delicate operation in heavy clothes in the cold and will encourage the use of the technique by those without cold-room facilities.

Another method that seems promising for quantitative work with injected insects consists of testing those insects inoculated with the more concentrated solutions singly and those inoculated with more dilute solutions in colonies of different numbers, depending on the concentration. From the proportion of infective colonies the percentage of infective insects can be estimated by calculation (14) and satisfactory data obtained for a series of dilutions that otherwise might require a much larger number of test plants. Such data are theoretically most accurate when 50 per cent of the colonies happen to be infective. Using these methods, we have studied the correlation between particles and activity in zones obtained from extracts of tumors caused by wound-tumor virus and in zones obtained from extracts of viruliferous insects. In both cases the results were the same and establish the identity of this

virus with a specific photographed particle with virtual certainty (14).

In our experiments with wound-tumor virus, the slope of a dilution curve derived from many experi-



ments with single insects was taken as a constant and was applied freehand to the data of individual experiments to give quantitative estimates of the virus concentrations. Possibly the average slope for many experiments in which colonies were tested would serve the same end. Such estimates are, of course, not so accurate as estimates derived from local-lesion determinations but they are practicable. There is need, however, for a statistical study of the validity of this procedure which we have based on the assumption that the large variation in slope from 1 individual experiment to another is largely due to chance and that the use of a constant slope based on many data from several experiments gives a truer estimate than would the measured slope of any single test.

Maramorosch (29, 30) has reported that the incubation periods of wound-tumor virus and aster-yellows virus in inoculated insects vary with the concentration of the virus injected, and has suggested the use of such variation for quantitative measurements of virus. An experimental comparison of this technique for measurements of infectivities with the technique of determining percentages of insects infected is also desirable. Perhaps a combination of these 2 methods would be more accurate than either one alone.

In an effort to reduce variation in results of injections, Storey (43) and Maramorosch (31) have used microsyringes for injecting uniform volumes of solution into each leafhopper. The use of a microsyringe adds to the difficulty of the technique and does not insure that a uniform dose will stay in the injected leafhopper since variable amounts of solution leak out on withdrawal of the needle. Although no study has been made to determine if the microsyringe technique gives less variable results than injection of approximately equal volumes by estimate of the operator, it would seem likely to do so and some simple means of uniform volume injections is much needed. Maramorosch (34) has devised an instrument which seems to provide a solution to this problem as long as one uses the same glass capillary for injecting. The breaking of a glass needle after performing a certain number of injections necessitates the use of a new needle which will almost certainly have a different aperture and therefore result in the injection of a different volume of solution. A sharp durable transparent needle fine enough for this work would be a great advantage over the present glass capillary.

Now that serological techniques have been applied successfully to a leafhopper-transmitted virus (7), it is clear that serological methods may provide very much quicker and less variable assays than do insect injections, and can be employed to great advantage in certain experiments. It is of interest to compare the attributes of these 2 techniques when applied to wound-tumor virus. We have obtained occasional infections with wound-tumor virus at dilutions of plant tumor extract at 10^{-5} and of insect extract at 10^{-4}. Although comparisons of the sensitivity of the serological and insect-injection techniques have not yet been made on the same solution, the serological titers of similar preparations have been around 1/320 or about $10^{-2.5}$ indicating the much greater sensitivity of the insect-injection technique. This high sensitivity and the small volumes sufficient for injection mean that the injection technique can be employed on very small volumes of concentrate and can be combined fruitfully with electron microscopy. However, consistent determinations of smaller differences in virus concentration can be obtained serologically in a matter of hours whereas the results from injections are not completed until many weeks have elapsed—12 weeks in the case of wound-tumor virus.

Although progress has been made with the potato yellow-dwarf, tomato spotted-wilt, and wound-tumor viruses, such progress is clearly just a beginning. All of these viruses are easier to work with than is the aster-yellows virus, the physical properties (5) of which help explain why it has not so far been demonstrated under the electron microscope. Once success has been attained with it, or with another of the yellows group, many others probably will be successfully attacked.

It has been our experience that with aster-yellows virus, infections can be obtained regularly with viruliferous insect juice diluted 10^{-3} but not 10^{-4}. With wound-tumor virus, infections can be secured quite regularly with viruliferous vector juice diluted 10^{-4}. If *Macrosteles divisus* is about as susceptible to inoculation with aster-yellows virus as is *Agallia constricta* to wound-tumor virus, then this may indicate an aster-yellows virus concentration in the vector about one-tenth that of the wound-tumor virus. It may well be that we now have techniques that would make the

FIG. 1–4. Labile insect-transmitted viruses, all magnified 30,000× to show their relative size. Fig. 1. Wound-tumor virus from plants. The large particles are polystyrene latex spheres, 260 mμ in diameter (Shadows 6× height). Fig. 2. Wound-tumor virus from leafhopper vector (shadows 3× height). In both pictures the outlines of some particles and shadows indicate that the dessicated virus is polyhedral. It is about 75 mμ across. (Fig. 1 and 2 after Brakke, Vatter and Black (14).). Fig. 3. Tomato spotted-wilt virus is larger (about 85 mμ across) than wound-tumor virus and also shows indications of a polyhedral shape for some virus particles (shadows 3× height) (After Black, Brakke and Vatter (8)). Fig. 4. New Jersey potato yellow-dwarf virus. The potato yellow-dwarf viruses are the largest plant viruses yet described. Their shape varies in different preparations; rarely are they as equidimensional as in this picture. In some preparations there are many more collapsed bodies, in others many more rod-like forms than are shown here (shadows 3× height) (Fig. 4 courtesy M. K. Brakke and A. E. Vatter).

detection of the aster-yellows virus possible in the near future.

Some of the best pictures of wound-tumor virus, of tomato spotted-wilt virus, and of potato yellow-dwarf virus we have so far obtained are presented in Figures 1–4. The purified viruses have been prepared and identified by techniques discussed in this paper and mounted for electron microscopy by a method devised by Vatter (44).

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SOME PLANT-VECTOR-VIRUS RELATIONSHIPS OF SOUTHERN CUCUMBER MOSAIC VIRUS[1]

John N. Simons

SUMMARY

Southern cucumber mosaic virus was transmitted experimentally by the cotton aphid, *Aphis gossypii* Glover; the green peach aphid, *Myzus persicae* (Sulz.); and the bean aphid, *Aphis rumicis* L., in that order of efficiency. The acquisition threshold period for both the cotton aphid and the green peach aphid was between 5 and 10 seconds.

An attempt was made to determine whether either of 2 species of plants (pepper and chard) used for rearing aphids could influence the ability of the aphid to transmit. Similarly, these species were compared as inoculum sources and as test plants. Pepper was the better host plant for aphids and the more susceptible test plant, whereas chard seemed to be a better source of virus. Use of the least compatible combination—chard as aphid host, pepper as inoculum source, and chard as test plant—resulted in a significantly lower level of transmission than did the converse—pepper as aphid host, chard as inoculum source, and pepper as test plant. Other combinations gave intermediate results.

The mean length of the initial naturally-terminated feeding time was 14.5 seconds. A maximum percentage of vectors acquired virus in this time. The presence of the acquisition threshold period was indicated by the low amount of transmission obtained in feeding periods of 10–12 seconds.

Southern cucumber mosaic (southern celery mosaic) long has been recognized as a severe disease of both celery and pepper in the Everglades area of south Florida. In recent years damage to celery has been negligible, but occasional economic loss in pepper still is found. The explanation for this possibly may lie in the better control currently maintained by the celery growers of weeds of *Commelina* spp. Doolittle and Wellman[2] first associated Commelina with field spread of the disease in Florida. They also showed that the cotton aphid, *Aphis gossypii* Glover, is a vector of the virus. The work reported here was done to learn more fully some of the insect relationships of southern cucumber mosaic virus.

MATERIALS AND METHODS.—Seedlings of celery, *Apium graveolens* L., var. *dulce* (Golden Supreme); pepper, *Capsicum frutescens* L. (California Wonder); and swiss chard, *Beta vulgaris* L., var. *cicla*, were used as test plants. Seeds were germinated in vermiculite and the young plants were transplanted to 4-in. clay pots.

The green peach aphid, *Myzus persicae* (Sulz.), was colonized on caged pepper or chard plants in the greenhouse. The bean aphid, *Aphis rumicis* L., was raised on purslane, *Portulaca oleracea* L., in the same manner. Considerable difficulty in rearing the cotton aphid on caged (shaded) celery plants was encountered. Use of unshaded celery plants was essential; eventually a technique of rearing this species in moist chambers on leaf cuttings of unshaded celery was developed. The reason or reasons for the unsuitability of shaded plants have not been established. Results suggestive of the same phenomenon have been reported for the pea aphid, *Macrosiphum pisi* (Kalt.)[3].

All aphid transfers were made by the camel's-hair brush technique. The insects were fasted in glass shell vials and feedings were timed with a stop watch. Plants were fumigated with nicotine before they were placed in the greenhouse and were routinely sprayed to control insects.

IDENTIFICATION OF THE VIRUS.—The original source of virus was a diseased plant of *Commelina* sp. growing near Belle Glade, Florida. The virus was maintained in Turkish tobacco (*Nicotiana tabacum* L.). Juice inoculations from Turkish tobacco to celery, cucumber, chard, tomato, pepper, and *N. glutinosa* L. induced symptoms similar to those described by Wellman[4] for this virus.

Property studies in which *N. tabacum* was used as both the source and the test plant indicated a thermal inactivation point of 65–70°C and a dilution end-point of 1:5000.

ACQUISITION THRESHOLD PERIOD.—Groups of adult, apterous cotton aphids and green peach aphids were fasted for 58–115 minutes ($\bar{x} = 76.1$ minutes) and then fed for periods of 5, 10, 15, 20 and 25 seconds on a diseased celery plant. They then were placed singly on healthy celery seedlings and given test feedings of 58–103 minutes ($\bar{x} = 78.8$ minutes). Each species-treatment combination was repeated twice at random in each of 15 replications. Ratios of the number of plants infected to the total number of plants fed upon for feeding periods of 5, 10, 15, 20 and 25 seconds were as follows: cotton aphid—0/27, 2/30, 1/28, 5/28,

[1] Accepted for publication December 2, 1954.
Florida Agricultural Experiment Station Journal Series, No. 325. The assistance of Mr. E. King in preparing the graphs is gratefully acknowledged.

[2] Doolittle, S. P., and Wellman, F. H. 1934. Commelina nudiflora, a monocotyledonous host of a celery mosaic in Florida. Phytopathology 24: 48-61.

[3] Barker, J. S., and O. E. Tauber. 1954. Fecundity of the pea aphid on garden peas under various combinations of light, moisture, and nutrients. Jour. Econ. Entom. 47: 113-116.

[4] Wellman, F. L. 1935. The host range of the southern celery mosaic virus. Phytopathology 25: 377-404.

7/28, respectively; green peach aphid—0/30, 1/30, 0/29, 2/28, and 0/29, respectively. When the figures for all periods were totalled, the ratios for the cotton aphid were 15/146 or 11 per cent and for the green peach aphid 3/146 or 2 per cent. (Several plants died, therefore the denominator is not always 30).

Although both species transmitted at the 10-second interval, the cotton aphid appeared to be the more efficient vector. In order to establish this point more clearly, the following experiment was done.

VECTOR EFFICIENCY.—Groups of noninfective adult, apterous cotton aphids, bean aphids, and green peach aphids (the latter were reared on either pepper or chard) were fasted for 67–119 minutes ($\bar{x} = 91.3$ minutes). Single insects were given an acquisition feeding period of natural length and then placed on pepper seedlings for test feedings of 66–158 minutes ($\bar{x} = 101.2$ minutes). Five cotton aphids, 5 bean aphids and 10 green peach aphids were used in each replication and 6 replications were carried out. The results were as follows: cotton aphid infected 11 of 29 plants; green peach aphid from pepper infected 9 of 27; green peach aphid from chard infected 3 of 26; and the bean aphid infected 3 of 28 plants. Both the cotton aphid and the green peach aphid seemed to be fairly efficient vectors. Differences in the ability to transmit of green peach aphids reared on chard and of those reared on pepper suggested that the host plant may influence the ability of aphids to act as vectors. The following experiment yielded information on this point.

Approximately 15 plants of pepper and of chard each were colonized with nonviruliferous green peach aphids. Macroscopically, there appeared to be some differences between the 2 colonies. Aphids on chard were more yellowish and more robust than those on pepper. Overall length was about equal, but the above characteristics made identification of aphids from either plant species relatively easy. After the colonies had become well established, 5 colonies on each plant species were selected and groups of adult apterae from each were placed in vials where they were fasted for 46–109 minutes ($\bar{x} = 79.7$ minutes). They then were given naturally-terminated, timed, acquisition feedings on diseased pepper and chard plants. Next, they were placed on either pepper or chard plants for test feedings of 55–101 minutes ($\bar{x} = 69.2$ minutes). All possible combinations of the above 3 variables were used; 5 aphids for each combination were used in each replication; and 12 replications were carried out. Order of use of the combinations in each replication was randomized and, in all, between 15 and 20 colonies of aphids from each species were used.

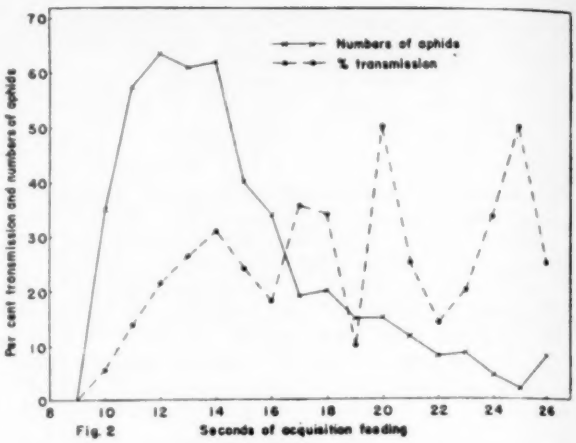

FIG. 2. Acquisition feeding times in seconds and percentage of transmission at each feeding time for green peach aphids and southern cucumber mosaic virus. The solid line indicates the number of aphids which fed at each time.

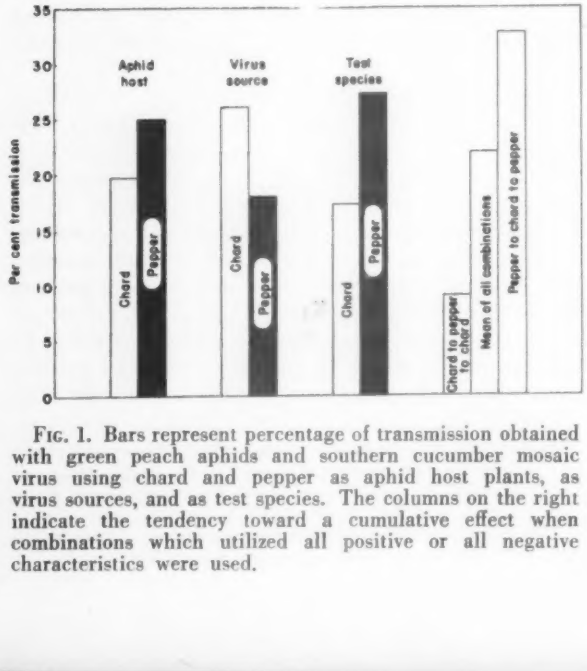

FIG. 1. Bars represent percentage of transmission obtained with green peach aphids and southern cucumber mosaic virus using chard and pepper as aphid host plants, as virus sources, and as test species. The columns on the right indicate the tendency toward a cumulative effect when combinations which utilized all positive or all negative characteristics were used.

Certain differences between chard and pepper as aphid host plants, as virus sources, and as test species were indicated (Fig. 1)[5]. These differences were least evident when the variable was the aphid host plant, and most apparent when it was either the virus source plant or the test species. A cumulative-type response was noticeable among the several combinations. If in the three combinations, the plant species are identified as H if a good host, h if a poor host; S if a good source, s if a poor source; T if a good test plant, and t if a poor test plant, the combination H-S-T gave the most transmission (33.3 per cent; $X^2 = 5.0$) and the combination h-s-t the least (8.8 per cent; $X^2 = 3.49$).

[5] Because of very low transmission the first 3 replications were discarded in the computation of the statistical data. Results are based on 45 plants in each treatment.

In the combinations where 2 of the 3 components were superior, i.e., H-S-t, H-s-T, and h-S-T, the mean amount of transmission was 25.9 per cent (S.D. = ±5.62 per cent); while in combinations where 2 of the 3 components were inferior, i.e., h-s-T, H-s-t, and h-S-t, the mean level of transmission was 19.2 per cent (S.D. = ±4.83 per cent). Although confirmatory data would be desirable, the trend seems evident.

Information on feeding habits of aphids also was obtained in this experiment (Fig. 2). The curve representing numbers of aphids feeding indicates that the length of time each aphid fed was generally 11–14 seconds (x = 14.5 seconds). Since each aphid had an approximately equal opportunity to feed, this "binomial type" response is not surprising. Transmission increased sharply up to 13–14 seconds of acquisition feeding, and then remained fairly constant. The low level of transmission during the 10–12 feeding period was evidently a reflection of the presence of the acquisition threshold period.

DISCUSSION.—It has been suggested that a partial explanation of differences in vector efficiency among species of aphids transmitting nonpersistent viruses could be due to a host–plant influence on the aphids.[6] Evidence presented here suggests that this type of interaction may exist but that it is relatively unimportant. The concept that the number of transmissions is influenced by the recipient plant is indicated by the data of Sylvester and Simons[7] and by the data reported here. Also, that the species of plant used as a virus source influences vector efficiency is evident. That these 3 factors can, in certain combinations, react to cause significant changes in vector efficiency is indicated by the results.

Southern cucumber mosaic virus would appear, from the limited evidence presented here, to be a typical nonpersistent aphid–borne virus.

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[6] Simons, J. N., and E. S. Sylvester. 1953. Acquisition threshold period of western celery mosaic virus for four species of aphids. Phytopathology 43: 29-31.

[7] Sylvester, E. S., and J. N. Simons. 1951. Relation of plant species inoculated to efficiency of aphids in the transmission of Brassica nigra virus. Phytopathology 41: 908-910.

NEW GERM PLASM FOR COMBINED RESISTANCE TO HELMINTHOSPORIUM BLIGHT AND CROWN RUST OF OATS[1]

H. R. Rosen

SUMMARY

A number of oat selections derived from a mutant of Tennessee 1922 × Bond-Iogold offer a new type of germ plasm for combined resistance to races 45, 57 and 101 of the crown rust organism and to the Helminthosporium blight pathogen. Most selections that have been saved possess a high degree of winter hardiness under Arkansas conditions. Under controlled experimental conditions, the unique type of crown rust resistance present in these selections is primarily associated with temperatures averaging below 70°F. At 60°F these selections exhibit an immune (I) type reaction while at 70°–76°F they are completely susceptible. Average temperatures are below 70°F during most of the winter oat growing season in Arkansas.

The appearance of new races of pathogens from time to time or of new and enlarged epidemics of older races on oats necessitates continual search for new germ plasm. It seems clear that the epidemics experienced on oats during the past decade were in part caused by the extensive use of varieties possessing the same germ plasm. It follows that the greater the diversity of germ plasm utilized in any one region, the less opportunity there is for any parasite new or old to induce the same host reactions. Therefore, the finding of new sources of germ plasm for resistance to major diseases is important.

A preliminary report of this work has been published.[2]

ORIGIN AND DESCRIPTION OF A NEW SOURCE OF GERM PLASM WHICH OFFERS RESISTANCE TO PREVALENT RACES OF PUCCINIA CORONATA CDA. AND TO HELMENTHOSPORIUM SATIVUM PAM., KING & BAKKE VAR. VICTORIAE (MEEHAN & MURPHY) ROSEN, WISER & YORK.—In 1946 a single rust-free plant was found in a field plot of R 22-36-9, Tennessee 1922 × Bond-Iogold, that was otherwise heavily rusted with race 45. This plant probably represented a mutation rather than an outcross since the types of morphological and physiological

[1] Accepted for publication December 9, 1954.
Research paper No. 1134. Journal Series, University of Arkansas. Published with the approval of the Director.

[2] Rosen, H. R. 1954. New germ plasm for combined resistance to Helminthosporium blight and races 45, 101, and allied races of crown rust of oats. Phytopathology 44: 504.

FIG. 1. Supernumerary florets in upper part of panicles, characteristic of numerous selections obtained from the mutation here described.

characteristics found in its progeny cannot be accounted for in any known parentage. The immediate progeny of this mutation was exceptionally variable in some pathologic reactions as well as in plant and seed characteristics. This variability continued for a number of succeeding generations. Out of 812 single panicle and single plant selections made in 1947, every promising selection found in 1948 duplicated the extreme variability noted previously. In any one selection there were plants varying in habit of growth, size of foliage, length and thickness of straw, winter hardiness, size of seed, color of lemmas, and smut resistance.

There are some characteristics, however, that have remained quite fixed: 1) supernumerary florets in the upper part of the panicle in many of the selections (Fig. 1); 2) large to medium-sized, spreading panicles; 3) a high degree of resistance to Helminthosporium blight, caused by *H. sativum* var. *victoriae*, to red spot mosaic and to yellow dwarf[3]; and 4) a high and unique type of resistance to races 45, 57, and 101 of *P. coronata* (new designations in part races 202, 203, and 213, respectively) under field conditions during most of the growing season.

This crown rust resistance represents a completely immune plant reaction (I type) to the races designated

[3] Rosen, H. R. 1952. Virus diseases of small grains in Arkansas, 1951-52. U. S. Dept. Agr. Pl. Dis. Rept. 36: 315-318.

FIG. 2. Left to right, 1 leaf of R22–75–102 (no infection); center leaf-Arkwin, about 5 per cent infected; 2 leaves of Lee, 20 per cent infected; all typical of each variety as seen in adjoining field plots on June 5, 1953.

and is comparable to the resistance shown by Bond and its derivatives to the old race 1.

It would appear that the mutation which occurred in a single plant of Tennessee 1922 × Bond-Iogold simply enlarged or perhaps multiplied the existing genetic factors originally present in Bond for resistance to many races of crown rust. It should be noted that in H. C. Murphy's identification and compilation of races of crown rust up to April 3, 1951, there were 85 races out of 101 to which Bond was resistant.[4] In M. D. Simons' most recent list (received July 31, 1954), involving a new set of differential varieties, Bond is shown to be resistant to 35 races out of 75, despite the fact that the rust collections were made at a time when Bond derivatives largely replaced Victoria derivatives over a considerable part of the country.

FIELD TESTING OF RESISTANCE. — Every year since 1946, when the mutation was first discovered, a severe epidemic has been initiated artificially in the nursery by 2 different methods: 1) by the inoculation of young tillers with heavy spore suspensions, usually of races 45 and 57, by means of large hypodermic syringes and needles, and 2) by the transplanting of severely rusted plants from the greenhouse to the nursery. Every tenth row in the nursery was planted to the rust-susceptible variety Lee, so that in most years there was abundant rust present. Under such conditions most of the selections derived from the mutant remained completely free from crown rust (Fig. 2) up to the early stages of maturity whereas Lee often had as much as 80-100 percent of the leaf area affected (modified Cobb's scale). However, as the plants were reaching full maturity, usually in the first or second week of June at the University farm at Fayetteville, these selections would become completely susceptible, as evidenced by large, fully developed pustules. By that late date the grain yield was not affected.

RELATIONSHIP OF TEMPERATURE TO RESISTANCE AND

[4] Thanks are due Drs. H. C. Murphy and M. D. Simons for permission to use these data.

SUSCEPTIBILITY. — To determine the cause for the change from resistance to susceptibility, experiments were conducted primarily involving differences in temperature. Such differences were suspected as being the primary cause, since in repeated greenhouse tests all of these selections were found to be susceptible in the seedling as well as in later stages, despite their resistance in the field. In the greenhouse studies an attempt was made to keep the temperature at 70°F or above, and thermographic recordings show average fluctuations from 70°–76°F during the winter months. This was when most of the work was done.

Six pots of seedlings of each of 4 varieties or selections in the first leaf stage were inoculated with a mixture of urediospores of races 45, 57, and 101 (in part races 203 and 213, obtained from M. D. Simons). Each pot was thinned to 25 seedlings. The varieties or selections were Bond, Lee, R 22-64-30, and R 22-75-102, the last 2 being selections from the mutant. Three pots of each variety or selection were placed in a moist chamber in the greenhouse, and 3 were placed in an incubator maintained at 60°F. The plants which were to be kept at 60° were held at that temperature for 48 hours prior to inoculation. All seedlings were debloomed by hand.

The incubator was equipped with 2 fluorescent lights which provided 350 foot-candles at the level of the seedling leaves. To avoid drying of the inoculum, a transluscent, nonporous, plastic cover was left over the seedlings for 48 hours after inoculation, both in the greenhouse and in the incubator.

Seedlings of all varieties and selections kept at greenhouse temperatures of 70-76°F began showing uredia within 7 days after inoculation. Within 10 days all varieties and selections showed approximately 80 per cent of the inoculated leaf area with well developed pustules. All varieties and selections appeared equally infected and completely susceptible.

In the incubator kept at 60°F, no plants showed uredia until 12 days after inoculation. At the end of 14 days Bond and Lee showed numerous pustules while R 22-64-30 and R 22-75-102 showed none. At the end of 21 days, the last 2 remained free from infection whereas Bond and Lee were heavily infected, the number of pustules approximating that attained at temperatures of 70°–76°F. The plants kept at 60°F for 21 days then were placed in the greenhouse at the higher temperatures to bring out possible latent infections. None appeared during several weeks of observation.

DISCUSSION.—In a recent paper on crown rust, Simons[5] reported that, "with one exception, varieties differing in reaction were more resistant at the low temperature used, 15°C [58°F] than at the higher temperature, 25°C [76°F]." Four Bond derivatives were included but with these the temperatures utilized were not as important in inducing changes in reaction to race 45 (205) as was age of tissue.

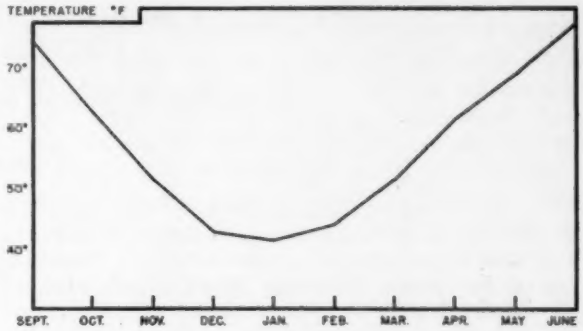

FIG. 3. Average Arkansas state temperatures during the growth of winter oats.

It seems clear that these 4 Bond derivatives and the selections here described differ in their reactions at different temperatures. Furthermore, there appears to be no general rule that applies to the influence of temperature on reaction of different germ plasm to crown rust race 45. The complete susceptibility of Bond at 60°F as well as at 70°–76°F, the immune reaction of the selections at 60°F here described, and a susceptible reaction of the same selections at the higher temperatures suggest that the effect of temperature is largely on the host rather than on the parasite. However, at the lower temperature, the period of incubation is considerably prolonged in susceptible varieties and the size of pustules somewhat reduced.

Under natural conditions in the field, temperatures seldom are uniform: the day and night temperatures often differ as much as 20°F. In Arkansas, winter oats are seeded in September or later and are harvested during the latter part of the following May up to the first half of June. Average temperatures in the state during this period are shown for each month in Figure 3.

During this 10-month period, average temperatures reach 70°F or above for about half a month in September when the seedlings are above ground, and for another half-month in June. These are the periods when the selections here described may be in a stage susceptible to crown rust. However, should temperatures rise above average, and this occasionally happens in the fall or late spring, it is quite likely that the susceptible periods would be correspondingly prolonged.

[5] Simons, M. D. 1954. The relationship of temperature and stage of growth to crown rust reaction of certain varieties of oats. Phytopathology 44: 221-223.

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THE ROLE OF SEEDLING YELLOWS AND STEM PITTING IN TRISTEZA OF CITRUS[1]

A. P. D. McClean and J. E. van der Plank

SUMMARY

Fraser demonstrated a consistent qualitative difference in the virus content of different species of citrus in orchards where tristeza occurs. She used, as test plants, seedlings on their own roots of Eureka lemon, sour orange, and grapefruit, and transmitted infection to them by grafting. The virus complex occurring naturally in sweet-orange and mandarin trees caused severe stunting and yellowing of the test plants. But when virus occurring naturally in grapefruit, Eureka lemon, and sour-orange trees was transmitted by budding into the above-mentioned test plants, there were either no leaf symptoms or just occasional vein fleckings in the spring growth, and no apparent stunting.

It is suggested that the tristeza virus complex has 2 components: a stem-pitting component, able on its own to cause stem pitting in grapefruit, and a seedling yellows component that by definition must also be present to cause severe stunting and yellowing of seedlings of Eureka lemon, sour orange, or grapefruit.

Sweet-orange and mandarin trees on their own roots escape severe injury because, although they are infected with the whole tristeza virus complex, they are reasonably tolerant of it; but on rootstocks like sour orange or grapefruit that are intolerant of seedling yellows they suffer severely after the virus complex has crossed the graft union. Eureka lemon and sour-orange scions (on their own or any roots) ordinarily escape severe injury under conditions of natural infection because they do not become infected with the whole complex, but only with a part, the stem-pitting component, to which they are fairly tolerant. Grapefruit as a scion also accepts the stem-pitting component with exclusion of the seedling yellows component; however, because of sensitivity to this component, grapefruit shows symptoms of decline, doing so relatively slowly on its own roots but rather faster on sour orange.

In Australia, Fraser[2] demonstrated that, when exposed to tristeza infection, Eureka lemon, grapefruit, and sour orange were infected differently from sweet orange and mandarin; this difference is the starting-point of some suggestions by us about the relationship between tristeza and stem pitting and the reason for the different behavior towards tristeza of these 5 species, on their own roots or in different scion-stock combinations.

FRASER'S EXPERIMENTS.—In an area in which tristeza occurs and tristeza-tolerant rootstocks are used, Fraser made a survey of trees of various species and varieties, of as wide a range of age as possible. She cut from the tree to be tested an axillary bud and grafted it onto a decapitated seedling on its own roots. The grafted plant was then allowed to send out lateral seedling shoots and in these shoots the symptoms were observed. The test seedlings were of various species, including Eureka lemon, sour orange (mostly bittersweet), and Marsh grapefruit, and a minimum of 5 seedlings of each species was used in each test. West Indian type lime seedlings were also used.

When virus was transmitted in this way from infected sweet-orange trees to Eureka lemon seedlings, the growth of the new shoots of the seedlings ceased after 3–6 leaves had been formed. The leaves were reduced in size and yellow, at first marginally and finally over the whole blade. Infected seedlings were still in this "shock" phase without signs of recovery for the 15 months they were under observation. These symptoms are those of seedling yellows, as Fraser named the disease. Symptoms in seedlings of sour orange and Marsh grapefruit were very much like those in Eureka lemon.

The species of Citrus in the grove found to contain virus that produces seedling yellows in the seedling test plants were sweet orange (7 varieties, 84 trees), mandarin (7 varieties, 35 trees), calamondin (1 tree), and rough lemon (1 tree).

However, when buds were taken from grapefruit trees and grafted onto Eureka lemon, sour-orange, or grapefruit seedlings they failed to produce seedling yellows. There were either no leaf symptoms or just occasional vein fleckings in spring growth, and no apparent stunting.

Of the trees surveyed, sour orange (4 varieties, 52 trees), Eureka lemon (7 trees), shaddock (3 trees), and citron (2 varieties, 2 trees) behaved like the grapefruit trees (5 varieties, 61 trees).

Of the grapefruit trees tested and referred to in the previous paragraph, the majority showed symptoms of stem pitting. Of the sour orange trees tested, 2 were trees with severe symptoms of stem pitting.

[1] Accepted for publication December 6, 1954.

[2] Fraser, L. 1952. Seedling yellows, an unreported virus disease of citrus. Agr. Gaz. of New South Wales 63: 125-131.

Fraser herself makes no mention of stem-pitting symptoms in her test plants. It might therefore be helpful to interpolate here that in Pretoria, where work follows the same pattern as Fraser's, stem pitting is a constant symptom in grapefruit seedling test plants, irrespective of whether the source of infection is sweet orange, mandarin, grapefruit, or Eureka lemon, i.e., irrespective of whether it is accompanied by seedling yellows or not.

The results listed by Fraser were for trees known, by inoculation to West Indian type lime seedlings, to be naturally infected with a virus. It might be explained here that West Indian type lime seedlings are good test plants for determining simply whether virus is present or not. Irrespective of whether the source of virus is naturally-infected sweet orange, mandarin, grapefruit, Eureka lemon, or sour orange, West Indian lime reacts with symptoms of stem pitting and vein flecking. To some extent, West Indian lime also reflects qualitative differences in the virus from different species. As recorded by Fraser, the presence of seedling yellows usually produces an intensification of the symptoms in the West Indian lime, followed later by considerable corking of the veins and splitting of the stem. We have however found that this type of reaction is not necessarily specific and may develop occasionally even in the absence of yellows. In this detail our findings differ from Fraser's.

Fraser records that, in almost every year, aphids were active in spring and autumn, and her inference was that trees of species like sour orange, Eureka lemon, and grapefruit, which do not have seedling yellows, are resistant to infection with it by aphids. This was confirmed by her in tests in which *Aphis citricidus* (Kirk.) was transferred from infected Washington navel orange and Emperor mandarin to Eureka lemon seedlings, 12–15 aphids being used in each transfer experiment. She obtained only temporary and localised seedling yellows infection, and buds taken from the new growth of 5 such seedlings failed to reproduce the disease on young Eureka lemon seedlings.

SEEDLING YELLOWS A SYMPTOM OF INFECTION BY THE TRISTEZA VIRUS COMPLEX.—The failure of buds from grapefruit and sour-orange trees with stem pitting to produce seedling yellows in seedlings of Eureka lemon and sour orange used as test plants was taken by Fraser to mean that seedling yellows is caused by a new virus "which is distinct from the tristeza-stem pitting complex". She added that "in nature, however, it has not yet been found unaccompanied by the tristeza virus".

But an example will show that Fraser had found no new virus and that seedling yellows is caused by a tristeza virus complex. Suppose a bud was taken from a tristeza-infected sweet-orange tree and put on a sour-orange seedling on its own roots. If the bud was allowed to grow out to form a shoot and all new growth of sour-orange shoots was removed, the result would be a sweet-orange scion on sour-orange rootstock; and the combination would soon suffer from tristeza disease. But suppose sour-orange shoots were allowed to grow. The leaves on the new sour-orange growth would turn yellow and the growth itself soon cease. This is seedling yellows disease under exactly the conditions in which Fraser saw it. But whatever course growth was allowed to take, the infective agent remains the same. If it is the tristeza virus complex when the sweet-orange bud is allowed to develop, it is also the tristeza virus complex when sour-orange shoots are allowed to grow.

STEM PITTING AND THE TRISTEZA VIRUS COMPLEX.—Seedling yellows is caused by tristeza virus complex. Fraser was unable to produce seedling yellows in appropriate test seedlings by grafting them with buds from grapefruit and sour-orange trees affected by stem pitting; so stem-pitting virus is not the whole tristeza virus complex, even if it is connected with it in some way.

Costa, Grant, and Moreira[3] were the first to suggest a connection between tristeza and stem pitting. In their own words, "it is probable that the tristeza virus that occurs in Africa is present in plants with stem-pitting, either being its only cause or being a component of a composite virus disease that would include another virus". We read the second alternative to mean that tristeza virus might be part of a stem-pitting virus complex.

With Costa, Grant, and Moreira's central idea of a connection between tristeza and stem pitting we agree; the evidence for it is strong. But we must reverse the detail, and interpret Fraser's results to mean that stem pitting is caused by a component of the tristeza virus complex.

Our suggestion is that tristeza is caused by a virus complex. One part of this complex, the stem-pitting component, is capable alone of causing the stem-pitting disease in grapefruit. The other part, the seedling yellows component, when present in addition to the stem-pitting component, causes seedling yellows in seedlings of sour orange, Eureka lemon, and grapefruit. The stem-pitting component can be isolated pure from the complex by the dropping of the seedling yellows component, e.g., during transmissions by aphids to grapefruit. But no way of filtering out the stem-pitting component from the complex to isolate the seedling yellows component has been found. Many inoculations and sub-inoculations made in the course of surveying the viruses of South African citrus have been watched for some sign that the stem-pitting component had by chance been lost to leave the other component pure, but in vain.

Nothing seems to have been published to allow it to be decided whether the 2 components are strains of a single virus or whether they are distinct viruses or even distinct virus complexes.

[3] Costa, A. S., T. J. Grant, and S. Moreira. 1950. A possible relationship between tristeza and stem-pitting disease of grapefruit in Africa. Cal. Citrograph 35: 504, 526-528.

SOME ASPECTS OF THE DECLINE OF CITRUS TREES ON THEIR OWN ROOTS AND IN DIFFERENT SCION-ROOTSTOCK COMBINATIONS. — From experience with tristeza in several continents, some generalizations have been distilled. Sweet-orange, mandarin, sour-orange, and Eureka lemon trees on their own roots generally escape serious harm. Grapefruit trees on their own roots decline with symptoms of stem pitting; the decline is slow and if the trees are well fed they may continue to produce profitable crops for 10–15 years or more after infection. Sweet orange and mandarin trees on sour-orange or grapefruit rootstocks decline swiftly and usually become worthless within a few years of infection. Grapefruit on sour-orange rootstock declines more slowly than sweet orange or mandarin on this rootstock. These findings are readily explained by our interpretation of Fraser's results.

The essence of the interpretation is the concept of tristeza virus as a complex that is ordinarily taken up as a whole by certain species of trees in the orchard, like sweet orange or mandarin, as a result of aphid transmission, but only in part by others, like grapefruit, sour orange, or Eureka lemon. Sour-orange and Eureka lemon trees generally escape severe injury because they take up only the part of the complex that they can tolerate. They are equally safe on their own roots or as scions on any rootstocks. Sweet orange and mandarin are safe enough on their own roots because they are fairly tolerant of the whole virus complex, but suffer on rootstocks like sour orange or grapefruit that are intolerant of seedling yellows after the complex has passed down across the graft union. We assume here nothing more than that species like sour orange or grapefruit, which as seedlings were shown by Fraser to react severely to seedling yellows when the complex is transmitted to them by grafting, would also react severely as rootstocks when the complex is transmitted to them from a scion across a graft union. Grapefruit as a scion escapes the full complex, but does not altogether escape injury because it takes up and reacts with the stem-pitting component, slowly on its own roots and rather faster on sour orange, which suggests that sour orange is possibly somewhat more sensitive to the stem-pitting component of grapefruit than grapefruit itself.

Bennett and Costa[4] explain matters differently. To give examples, they regard sweet orange as dangerous and Eureka lemon as safe on sour orange rootstock because the sweet orange allows the tristeza virus, or the toxin produced by the virus, to multiply in the scion to a level that is dangerous to the rootstock, whereas in Eureka lemon multiplication to a dangerous level does not occur.

Bennett and Costa see the explanation largely in a quantitative difference in the virus content of different species. We see it in the qualitative difference demonstrated by Fraser 3 years after Bennett and Costa wrote.

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[4] Bennett, C. W., and A. S. Costa. 1949. Tristeza disease of citrus. Jour. Agr. Res. (U.S.) 78: 207-237.

ELIMINATION OF SPOTTED WILT FROM DAHLIAS BY PROPAGATION OF TIP CUTTINGS[1]

Francis O. Holmes

SUMMARY

A clone of the dahlia variety Rhythm, all plants of which were severely affected by spotted-wilt disease, was transformed into a healthy stock of plants by the rooting of small stem-tip cuttings. Several other dahlia varieties were similarly freed from this disease. Apparently the causative virus does not move freely toward the growing tips of dahlia plants but lags behind the growth of the stem. Recovered stocks remained free of disease when grown in the absence of a source of reinfection.

During an outbreak of spotted-wilt disease in dahlias (*Dahlia pinnata* Cav.) several years ago, it was noted that all locally available plants of the variety Rhythm had become infected. The circumstances surrounding this outbreak have been described elsewhere (8), in connection with a study of resistance to spotted-wilt disease in tomato (*Lycopersicon esculentum* L.). The present paper describes experiments in the course of which the diseased clone was transformed into a healthy stock of dahlia plants by vegetative propagation of virus-free tips of stems.

Infected dahlia plants constitute a reservoir of spotted-wilt virus (*Lethum australiense* H.) from which spread may occur to nearby tomato plants and other

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susceptible crops or weeds. In the eastern United States, dahlias often seem to be the only persistent source of this virus. Control of spotted-wilt disease in them is therefore of primary importance because it tends to protect both healthy dahlias and other susceptible plants that may be in the vicinity. This raises the question of the best method, or methods, for control of spotted-wilt in the dahlia. One of the most commonly applied methods for control of viral diseases of plants in general is the removal and destruction of diseased individuals. This procedure would be very expensive as applied to dahlia, partly because of the relatively high value of each plant unit and partly because of the possible loss of varieties in instances of complete infection.

It seemed desirable to seek a more efficient method for control of the disease. This was done, using as experimental material the completely infected variety Rhythm.

Some viral diseases of plants can be cured by the application of moderate heat (11, 12, 13). A preliminary attempt was made to destroy spotted-wilt virus in dormant, overwintering plants of the Rhythm dahlia by hot-water treatment of roots and crowns, because it was known that this virus has a relatively low thermal inactivation point in expressed juices of such plants as the tomato (3). It was found, however, that diseased crowns of dahlia did not stand heating at all well. In consequence of this, the attempt to cure plants by heat treatment was abandoned in favor of another procedure, already reported in a brief abstract (7).

Several investigators have obtained healthy stocks of vegetatively propagated plants previously infected with viruses, by taking advantage of imperfect distribution of the viruses in the tissues of their hosts (1, 2, 4, 5, 9, 10, 14, 15). Patterns on leaves of plants affected by many viral diseases suggest that the distribution of the causative viruses may be discontinuous. This is true especially in mosaic-type diseases and in the early stages of ringspot-type diseases, when primary and secondary lesions are often discrete and when a tendency to show less and less damage in progressively younger tissues can be discerned. To what extent it may be true in the late stages of chronic ringspot diseases and yellows-type diseases is not yet known.

Spotted-wilt in dahlia produces ringspot-like patterns that are suggestive of a discontinuous distribution of virus in the tissues. It is not uncommon to find only one or a few concentric-ring patterns on the leaves of an infected plant. Even when lesions are numerous, young leaves generally show decidedly fewer lesions

FIG. 1. Leaves of Rhythm dahlia: A) from a diseased plant in the field, showing chlorotic spots, circles, and concentric markings characteristic of the stock when affected by spotted-wilt disease; B) from the same stock after its restoration to health. Photograph by J. A. Carlile.

than appear on old leaves of the same plant.

The Rhythm dahlia seemed more susceptible than most dahlia varieties to extensive injury by spotted-wilt. Its affected leaves tended to exhibit large numbers of lesions (Fig. 1, A). These lesions were conspicuous chlorotic or necrotic rings and concentric-line patterns. Necrotic spots and flecks were noted in diseased stems and roots. Even in this unusually susceptible variety, leaves near the top of the plant showed lesions distinctly less numerous than those on lower and older foliage. This suggested that the tips of rapidly growing stems might sometimes be free of spotted-wilt virus.

Overwintered plants of the Rhythm dahlia were grown in a greenhouse bench. Developing shoots were kept under observation to determine their state of health or disease from day to day. Frequently the shoots seemed healthy at first. In all observed instances, however, they became diseased later if left attached to the diseased roots. Earlier, Brierley (6) had noted a tendency toward spontaneous recovery in stocks of dahlia affected with the disease that he called dahlia ringspot. This disease is now regarded as spotted-wilt in dahlia. Permanence of the recovery observed by Brierley was not obvious. Reappearance of the disease was regarded as erratic, as indeed it would be at best if old stems and roots were left attached to growing plants indefinitely.

In the present work, cuttings made from the tips of young stems appeared in most cases to be free from disease and remained healthy in appearance after they were rooted. The few that were obviously affected were discarded. Under springtime conditions, many of the healthy-appearing rooted cuttings were grown to blossoming in a greenhouse without relapse.

As a further test of the completeness of recovery of the formerly diseased stock of the Rhythm dahlia, about 50 healthy-looking rooted cuttings were grown throughout the summer in a garden plot near Princeton, New Jersey, where spread of spotted wilt had not been noted in previous years. The plants grew well in most instances and no cases of spotted-wilt disease were noted during the entire growing season. The necrotic flecks observed the preceding year in diseased stems and roots of this variety were absent. Leaves remained free from the chlorotic and necrotic concentric rings formerly characterizing the stock (Fig. 1, B).

The present study indicates that spotted-wilt virus does not move freely toward the growing tips of Rhythm dahlia plants but lags considerably behind the growth of the stem. A fully diseased stock of this variety can be transformed into a virus-free clone of plants by the cutting away of all obviously diseased portions, propagation from healthy-appearing young tip-cuttings, and the discarding of any young rooted cuttings that subsequently show the disease.

Several other varieties of dahlia, including Pink Giant and Mab, also were freed from spotted-wilt disease by the rooting of small cuttings from tips of diseased plants. The unusually large proportion of leaves showing ringspots in infected plants of the variety Rhythm suggested a maximum of viral movement toward the growing point in this variety. This led to the supposition that all, or nearly all, other varieties of dahlia could be restored to health by similar treatment, even if every known stock of each of them should become infected. Stocks can be maintained free of the disease by growing them either in the absence of a source of the causative virus or in the absence of the vector insects.

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HELMINTHOSPORIUM LEAF STREAK OF TIMOTHY[1]

J. H. Graham

SUMMARY

A foliar disease of timothy characterized by brown, elongated lesions with chlorotic borders, was studied and the causal agent identified as *Helminthosporium dictyoides* Drechsl. var. *phlei* var. nov. The fungus is differentiated from *H. dictyoides* by spore morphology, production of secondary conidia, and host reaction. The Helminthosporium from timothy infected only timothy. *Helminthosporium dictyoides* and closely related species did not attack timothy. The optimum temperature for mycelial growth was between 24° and 28°C; for infection and disease development, between 20° and 24°C; and for sporulation on host tissue, between 15° and 24°C.

A previously undescribed leaf streak disease of timothy (*Phleum pratense* L.) caused by a species of Helminthosporium has been observed in Central Pennsylvania since 1950 and more recently in other Northeastern States. The pathogen differs from *H. giganteum* Heald & Wolf, *H. sativum* Pam., King & Bakke, and *H. tritici-vulgare* Drechsl., which have been reported to attack the foliage of timothy[2]. It also differs from morphologically related species of *Helminthosporium*.

SYMPTOMATOLOGY.—The disease on timothy leaves is characterized by irregular, light-brown necrotic streaks with conspicuous chlorotic borders. These necrotic areas (1-5 mm wide) are often marginal and may extend the length of the blade. Coalescence of lesions causes browning of most of the leaf, in which case chlorosis is confined to the basal end. In some cases the streaks may be narrow and the chlorotic symptom lacking. The disease is then difficult to distinguish from brown stripe, caused by *Scolecotrichum graminis* Fckl. Under moist conditions, however, the latter disorder is characterized by distinctive rows of dark tufts of conidiophores. Young lesions of the streak disease, as observed from artificial inoculations, are first small, chlorotic, and irregular in outline. They later develop light brown centers and elongate rapidly.

THE PATHOGEN.—The fungus sporulates on lesions, is isolated readily from surface-sterilized leaf pieces, and upon reinoculation produces symptoms similar to those found in nature. Brown conidiophores arise singly between epidermal cells or from stomates and, according to the environment, are 40–150μ (to first scar) × 6–9μ with 2–8 septa. Conidia are subhyaline to light brown, obclavate, straight, widest at basal or second cell, tapering uniformly to the apical cell (Fig. 1, B). The tip of the elongated apical cell usually is slightly enlarged. Conidia are 38–118μ × 10–18μ (usually 72–88μ × 12–15μ) with 2-7 septa (usually 4–5). There may be a slight constriction at the first septum. The contour of the basal cell is hemiellipsoidal, or rarely, hemispherical, and the apical end usually is hemispherical. The peripheral wall is thin (approximately 2μ) and includes the hilum within its contour.

Following several days in a moist atmosphere, secondary conidia develop from the tip of the apical cells of the primary conidia or the apical ends elongate and form septate conidiophore-like structures before the spores develop. Secondary conidia range from 93 × 14μ with 4 septa to 22 × 12μ with 2–3 septa. Germination occurs from the end segments and from 1 or more intercalary cells. Germ tubes arise laterally from the basal cells and usually terminally from the apical cells.

On potato-dextrose agar the fungus produces dense, raised, light- to iron-gray colonies. Conidial production is sparse on various agar media, but most isolates produce a moderate number of short conidia on autoclaved culms of timothy. The optimum temperature for mycelial growth is between 25 and 28°C. The sexual stage of the fungus has not been found, although sclerotia-like bodies have been observed in old cultures.

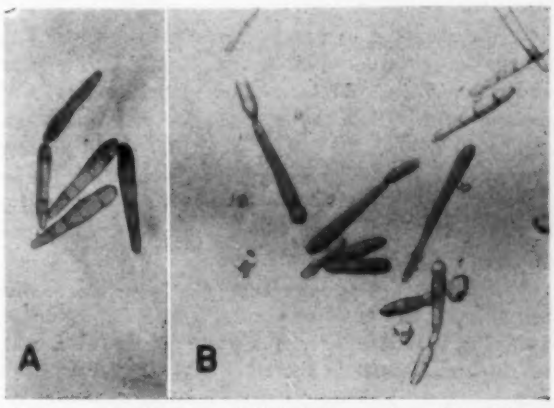

FIG. 1. A) Conidia of *Helminthosporium dictyoides* on meadow fescue. B) Conidia of *H. dictyoides* var. *phlei* on timothy.

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Contribution No. 139 of the U. S. Regional Pasture Research Laboratory, Field Crops Research Branch, ARS, USDA, State College, Pa., in cooperation with the 12 Northeastern States.

[2] Weiss, F. 1950. Index of plant diseases in the United States. U. S. Dept. Agr. Pl. Dis. Survey Spec. Publ. 1, part 3.

TABLE 1.—*Spore size and septation of Helminthosporium spp.*

Fungus	Host	Noted by	Size (μ)		Septa	
			Range	Average	Range	Average
H. dictyoides	Meadow fescue	Drechsl.	23–115 × 14–17	(50–70)	1–7	(3–5)
H. dictyoides No. 1	Meadow fescue	Graham	72–109 × 13–17	92.2 × 15.0[a]	4–7	5.4
H. dictyoides No. 2	Meadow fescue	Graham	103–122 × 15–18	108.6 × 16.0	5–6	5.6
H. dictyoides phlei No. 1	Timothy	Graham	43–118 × 11–18	88.1 × 13.7	2–7	4.7
H. dictyoides phlei No. 2	Timothy	Graham	38–113 × 10–17	79.8 × 13.6	3–6	4.1
H. catenarium	Woodreed	Graham	98–204 × 14–17	153.9 × 15.8	3–6	4.8
H. catenarium	Woodreed	Drechsl.	30–200 × 14–18	.....	1–10	—
H. catenarium	Sloughgrass	Nisikado	45–155 × 13–20	77.6 × 17.3	2–10	4.7

[a] 50–100 Conidia measured in collections made by Graham.

The Helminthosporium described above bears some resemblance to *H. catenarium* Drechsl. in the production of catenate spores. Spores of the latter fungus found on dead leaves of *Cinna arundinacea* L. were much longer (Table 1) than spores produced by the fungus from timothy, and were curved or bent in an irregular manner near the centers as described by Drechsler[3]. Spores from the organism on timothy developed more color and had a thicker exospore than those from *C. arundinacea*. On potato–dextrose agar *H. catenarium* produced powdery, white to dirty white, raised colonies with irregular margins.

Conidia from diseased timothy leaves resembled those of *H. dictyoides* Drechsl. on meadow fescue in general form, and in accordance with Drechsler's original description in size and septations (Table 1). However, spores of *H. dictyoides* produced on leaves of meadow fescue adjacent to diseased timothy were larger and had more septa (Fig. 1, A). The apical cells of the conidia were not bulbous, secondary spores were not produced, and germ tubes usually arose laterally from the end segments only. Furthermore, the conidiophores were dark brown to olivaceous and varied greatly in size.

PATHOGENICITY.—Artificial inoculations were made to determine the host range and the optimum temperature for infection and disease development. Inoculum was prepared by grinding leaf tissue on which the fungus was sporulating and, also, from cultures grown on a wheat-oat mixture. Inoculated plants were covered with "Polyethylene" bags for 3 days and incubated at 16°, 20°, 24°, or 28°C. Most damage occurred at 20° and only slightly less at 24°. When pieces of artificially inoculated leaves were placed in a moist Petri dish at temperatures from 5–28°C, greatest sporulation occurred at 15–23°.

Helminthosporium catenarium has been reported on Sudangrass, *Cinna arundinacea*, and on *Beckmannia syzigachne* (Steud.) Fernald while species of *Festuca* are the only reported hosts of *H. dictyoides* in the United States.[2] In the present study, timothy, meadow fescue, barley, oats, Sudangrass, *C. arundinacea* and *B. syzigachne* were inoculated with mycelial suspensions of *H. catenarium* and with conidial as well as mycelial suspensions of 3 collections of *H. dictyoides* and of the Helminthosporium from timothy. Twelve or more plants of each species, both seedlings and older plants, were inoculated in 4 separate experiments.

The timothy isolates were pathogenic only on timothy, whereas *H. dictyoides* infected only meadow fescue. None of the other plants tested proved to be a host to either organism. Plants inoculated with mycelial suspensions of *H. catenarium* did not become diseased. Of the many sources of timothy tested, individual plants varied from moderately resistant to very susceptible.

IDENTITY OF THE FUNGUS.—In view of the evidence presented herein, it is proposed that the Helminthosporium which attacks timothy be designated as a new variety of *H. dictyoides*. The proposed name and description follow:

Helminthosporium dictyoides Drechsl. var. **phlei** var. nov.—Strias necroticas irregulares 1-5 mm latas, saepe longitudinem totam laminae extensas efficiens; conidiophora simplicia, brunnea, ex stomatibus vel intra cullulas epidermicales singula oriunda, 40–150 × 6–9μ, 2–8 septata; conidia subhyalina usque pallide brunnea, obclavata, recta, ad cellulam basalem vel secundam latissima, gradatim apicem versus attenuata, 38–118 × 10–18μ (med. 84.2 × 13.6μ), 2–7– (med. 4.4–) septata, ad septum primum subconstricta, basi hemiellipsoidea, rare hemisphaerica, apice plerumque leniter subbulboso, pariete tenui, 2μ crasso, hilo conspicuo incluso; conidia secondaria e cellula apicali vel directe vel ex elongatione conidiophoroidea enata; germinatio e segmentis terminalibus et intercalariis, tubulis e cellula basali lateraliter, apicali terminaliter oriundis.

Hab. in foliis Phlei pratensis parasiticus, Pennsylvania.

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A METHOD OF FORECASTING DECAY IN CALIFORNIA STORAGE GRAPES[1]

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SUMMARY

Fumigation of California table grapes with sulfur dioxide surface-sterilizes them and thereby reduces losses from decay in storage, but it does not eliminate decay because the gas does not kill fungi that have already gained entrance into berries before harvest. Most of the decay in stored grapes, in fact, results from incipient infections not evident at harvest.

The percentages of incipient infections in Emperor grapes were determined following surface-sterilization of the berries by fumigation with sulfur dioxide and incubation at room temperature under aseptic conditions for 10 days. The percentages of decay that developed in grapes from the same lots after storage for 2½–4 months also were determined. These percentages were found to be closely correlated (average $r = 0.95$). Therefore, the laboratory determination could be used to forecast the decay likely to develop during storage. Grapes likely to develop decay could be sold early and those likely to remain sound could be stored safely.

Cladosporium herbarum Lk. ex Fr. and species of *Alternaria* and of *Stemphylium* were demonstrated to be the more important decay causing fungi in the early part of the harvest season, while *Botrytis cinerea* Pers. ex Fr. was found to be the chief cause of decay in the late part of the harvest season after periods of high humidity or rainfall.

Some varieties of California table grapes are held in cold storage for as long as 6 months. During this period they are fumigated at regular intervals with sulfur dioxide gas [2,3,4] to control decay caused by *Botrytis cinerea* Pers. ex Fr., *Cladosporium herbarum* Lk. ex Fr., and species of *Alternaria* and *Stemphylium*. Laboratory tests show that fumigation will surface-sterilize the fruit but will not kill organisms which already have gained entrance into the berry. Decay in storage results primarily from incipient infections that occur in the vineyard and have not advanced far enough to be detected during the packing operation. During storage these infections develop further and under some conditions the causal organism may spread by contact from infected to sound berries.

A method of estimating incipient field infections at harvest would provide a means of forecasting the amount of decay that would develop in a particular lot of grapes during storage. Such a forecast would improve marketing greatly as fruit with a high decay potential could be marketed soon after harvest and only fruit in which decay was not likely to develop would be held in storage for late marketing. A laboratory method for determining the amount of incipient decay in different lots of grapes at harvest is described in this paper. From the incipient decay found it was possible to predict how much decay would develop in storage.

METHODS.—Six randomized plots, consisting of 9 Emperor grape vines each, were selected in each of the vineyards for which forecasts were to be made. The Emperor grape was used because it is the principal storage variety grown in California. Approximately 25 lb. of fruit was harvested from each plot at weekly intervals (Table 1) during the period when grapes were being harvested commercially.

The laboratory test or forecast.—Injured berries were removed from the clusters and 120 apparently sound berries were removed at random from the entire harvest from each plot for a laboratory determination of incipient field infection. These berries were placed in glass storage dishes and were surface-sterilized by fumigation with a 1 per cent concentration of sulfur

TABLE 1.—*Harvest dates for stored Emperor grapes*

Harvest	Date of harvest from indicated vineyard		
	Fresno 1952[a]	Fresno 1953[b]	Dinuba 1953[c]
A	Oct. 7	Oct. 1	Oct. 2
B	Oct. 15	Oct. 6	Oct. 8
C	Oct. 21	Oct. 16	Oct. 15
D	Oct. 28	Oct. 23	Oct. 22
E	Nov. 5	Oct. 31	Oct. 30
F	Nov. 10	Nov. 6	Nov. 5
G	Nov. 20	Nov. 13	Nov. 12
H	Nov. 25	Nov. 19	Nov. 18

[a] All lots removed from storage Feb. 10, 1953.
[b] All lots removed from storage Feb. 4, 1954.
[c] All lots removed from storage Feb. 3, 1954.

[1] Accepted for publication December 7, 1954.

[2] Allen, F. W., and W. T. Pentzer. 1950. (Cold Storage of) apples, pears, and grapes. In The refrigerating data book. The Amer. Soc. Refrig. Engin. New York. p. 173-187.

[3] Jacob, H. E. 1929. The use of sulfur dioxide in shipping grapes. Calif. Agr. Exp. Sta. Bul. 471: 1-24.

[4] Winkler, A. J., and H. E. Jacob. 1925. The utilization of sulfur dioxide in the marketing of grapes. Hilgardia 1: 107-131.

dioxide gas for 30 minutes. After fumigation the dishes were covered and kept under aseptic conditions to prevent recontamination of the fruit. Twenty ml of sterile distilled water was added to each storage dish to provide the high moisture conditions favorable to development of decay organisms. For further protection against contamination, the dishes were wrapped in cellophane bags. The dishes of grapes were held at room temperature and the fruit was examined for decay after 10 days. As the sulfur dioxide fumigation had surface-sterilized the berries but had not killed fungus mycelium already within the fruit, the number of berries developing decay was a measure of incipient field infections. The percentage of infections in each lot was considered to represent the percentage of decay that would develop during storage.

The storage test.—The remaining fruit (about 20 lb.) of each harvest from each plot was immediately placed in storage at the accepted refrigeration temperature, 31–32°F. This fruit was fumigated initially with a 1 per cent concentration of sulfur dioxide gas for 20 minutes and during storage was treated at 14-day intervals with ¼ per cent concentrations of gas for 20 minutes. All fruit was removed from storage on the dates indicated in Table 1 and examined for decay.

Results.—In 1952, forecasts of decay were made for a vineyard near Fresno. The percentages of decay that occurred within 10 days in the grapes used in the laboratory test were closely correlated ($r = 0.95$) with the percentages of total decay that occurred in the storage samples of grapes when removed from storage on February 10 (Fig. 1, A). In both the grapes kept in the laboratory and those kept in cold storage there was a sharp increase in decay incidence in fruit harvested after November 10 and very little decay in fruit harvested earlier. Decay that occurred in grapes harvested before that date was caused chiefly by *Cladosporium herbarum* and *Alternaria* sp. In grapes harvested after November 10, the predominant type of decay was due to *Botrytis cinerea* (Fig. 2, A). Analysis of variance showed that the interaction between type of decay and harvest date was highly significant at the 5 per cent level.

In 1953, forecasts were made for the Fresno vineyard used in 1952 and for a vineyard near Dinuba, California. The vines used for the Fresno vineyard were the same as in the previous year. In the Fresno vineyard there was a close correlation between the percentages of total decay that developed in 10 days in the laboratory samples and the percentages that developed in storage (Fig. 1, B). However, such correlation did not occur in the Dinuba vineyard (Fig. 1, C) where the major portion of the decay was caused by species of *Alternaria* and of *Stemphylium* (Table 2). These organisms are relatively slow growing and apparently had not developed far enough in the laboratory test to be detected in 10 days.

If for the Dinuba vineyard only the percentages of storage decay caused by *B. cinerea* and *C. herbarum* are compared with the percentages of decay in the laboratory samples, there is a much closer agreement ($r = 0.92$) (Fig. 3, B). In the Fresno vineyard in 1953, species of *Alternaria* and *Stemphylium* accounted for only a minor portion of the decay (Table 2) and did not, therefore, cause such a pronounced discrepancy between the percentages of decay forecast and the percentages of total decay in storage. However, even in this vineyard there is a closer agreement

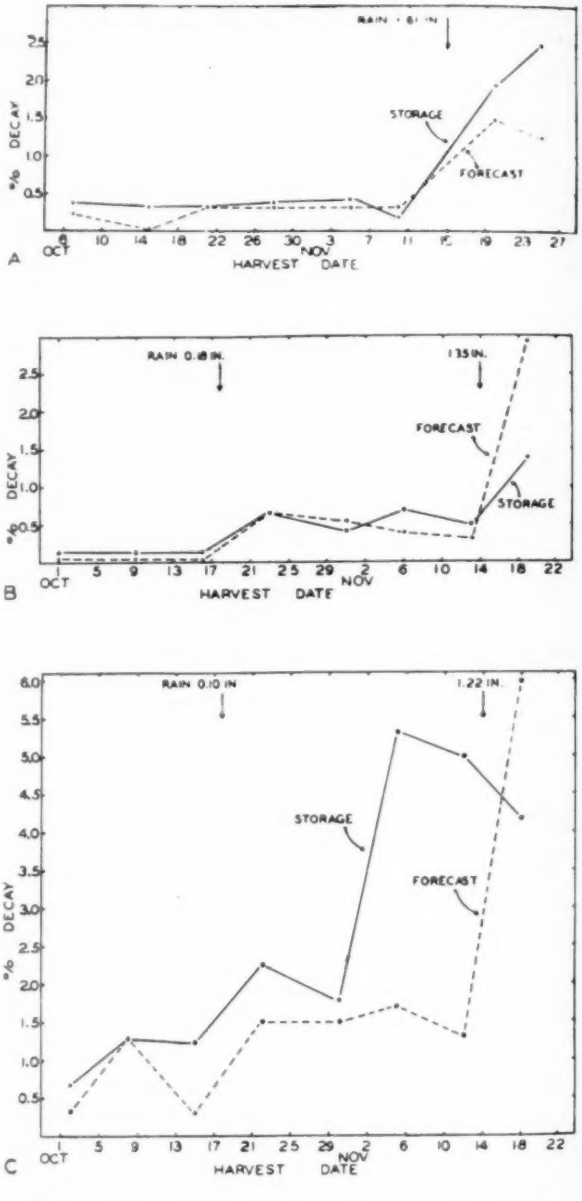

Fig. 1. Interrelation of harvest dates and decay forecast for and actually developing in stored Emperor grapes. A) Fresno vineyard, 1952. B) Fresno vineyard, 1953. C) Dinuba vineyard, 1953.

($r = 0.98$) between the forecasts and the percentage of storage decay caused by *B. cinerea* and *C. herbarum* than between the forecasts and the percentages of total storage decay (Fig. 3, A).

In both vineyards used in 1953, decay caused by *Botrytis cinerea* was slight early in the season, but it increased sharply after rains on October 18 and November 14 (Fig. 2, B and 2, C). Decay caused by *Cladosporium herbarum* did not fluctuate so much on the various harvest dates as did that caused by *B. cinerea*, nor did it rise so sharply after periods of rain. In the Dinuba vineyard (Fig. 2, C) decay caused by species of *Alternaria* and *Stemphylium* fluctuated greatly during the harvest season, but did not show a response to rain. In both vineyards, the interaction between type of decay and harvest date was highly significant at the 5 per cent level.

The effect of plot, or location in the vineyards, was significantly related to decay development in storage (Table 3). Most decay developed in fruit from Plot III in the Fresno vineyard in both 1952 and 1953. That in Plot V in the Fresno vineyard had the least decay in 1952 and only slightly more than the plot with the least decay in 1953. Essentially the same trend in decay with reference to plot was found in both 1952 and 1953 in the Fresno vineyard. In the one season in which the forecasts were made for the Dinuba vineyard there was also a significant difference in decay development in fruit from the different plots.

Discussion.—As all grapes used in the storage phase of these tests were removed from storage on the same date, lots picked early in the harvest season had been in storage longer than lots picked late in the season (Table 1). Although the early grapes were in storage 7 weeks longer than the late fruit, those harvested early had much less decay when they were removed from storage than did fruit harvested at the end of the

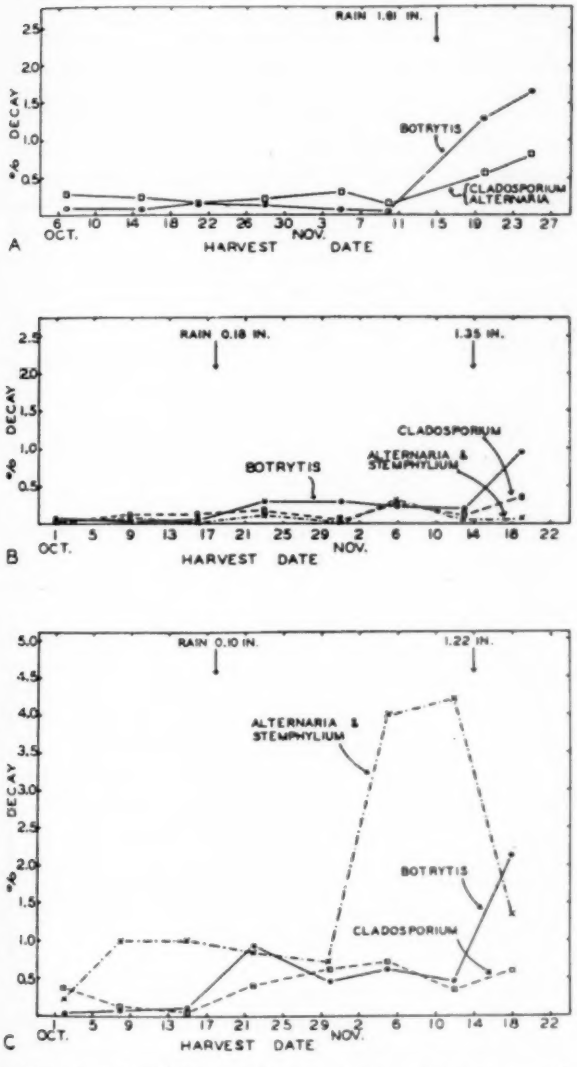

Fig. 2. Interrelation of harvest dates and decay caused by various fungi in stored Emperor grapes. A) Fresno vineyard, 1952. B) Fresno vineyard, 1953. C) Dinuba vineyard, 1953.

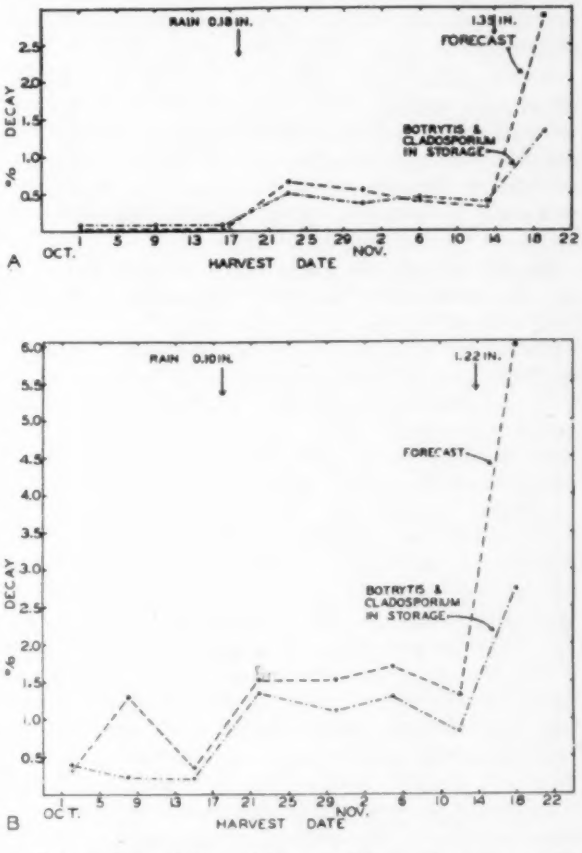

Fig. 3. Interrelation of harvest dates and decay caused by *Botrytis cinerea* and *Cladosporium herbarum* forecast for and actually developing in stored Emperor grapes, 1953. A) Fresno vineyard. B) Dinuba vineyard.

TABLE 2.—*Total decay caused by various fungi in storage grapes, 1952 and 1953*

Organism	Fresno vineyard, 1952	Fresno vineyard, 1953	Dinuba vineyard, 1953
	per cent	per cent	per cent
Botrytis cinerea	56.2	50.8	22.0
Cladosporium herbarum	43.8	30.5	15.3
Alternaria and *Stemphylium*		18.7	62.4

TABLE 3.—*Total decay of grapes from different plots, 1952 and 1953*

Plot	Fresno vineyard, 1952	Fresno vineyard, 1953	Dinuba vineyard, 1953
	per cent	per cent	per cent
I	1.01	0.63	1.81
II	0.61	0.57	3.14
III	1.19	1.11	2.83
IV	0.65	0.51	2.32
V	0.55	0.58	3.93
VI	0.72	0.68	2.22
L.S.D. 5%	0.32	0.18	0.95

season. These results provide a conclusive answer to the question whether grapes destined for the late market hold better on the vine or in storage. Length of storage has less effect on decay than the environment to which the fruit is exposed before harvest.

The relation between various types of decay in grapes and the harvest date is indicative of the infection requirements of the various organisms. Decay caused by *Botrytis cinerea*, for example, is related much more closely to rainfall than is decay caused by *Cladosporium herbarum* and *Alternaria* and *Stemphylium* spp. (Fig. 2). The curve in Fig. 2, C suggests that infection by *Alternaria* and *Stemphylium* spp. may be favored by periods of dry weather before rain, but this relation has not been confirmed by critical studies.

The excellent correlation between the amount of decay that develops from incipient infections within 10 days in laboratory samples of grapes and the amount of decay that develops in storage makes it possible to forecast which lots of grapes should be sold early to avoid excessive decay and which lots can be stored safely for longer periods.

Data for both the laboratory samples and the stored grapes indicate that more decay occurred in lots of grapes harvested after rainy periods than in those harvested earlier. When enough records have been obtained to determine the effect of rain, high humidity, heavy fogs, or morning dews, it may be possible to forecast from the weather records the keeping quality of grapes harvested at different dates. But as decay also varies from one vineyard to another and in different parts of a vineyard, determining incipient decay in laboratory samples probably will be the more nearly accurate way of forecasting decay development in specific lots of grapes. Laboratory determinations are not complicated and can be made at the packing house or storage plant level. By a careful selection of samples from representative vineyards served by the packing house or storage plant, accurate forecasts probably can be made under commercial conditions.

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STUDIES ON MECHANICAL TRANSMISSION OF THE YELLOWS VIRUS OF SUGAR BEET[1]

A. S. Costa and C. W. Bennett[2]

SUMMARY

Yellows virus was transmitted by juice inoculation but usually only to a small percentage of the plants inoculated. Infection resulted in the production of small chlorotic or necrotic lesions 10–20 days after inoculation. Production of local lesions was not followed by systemic infection in all instances. Evidence indicates that variety of plant inoculated, period of exposure of plant to low light intensities prior to inoculation, plant source of inoculum, strain of virus used, treatment of inoculum, and perhaps other factors, influence transmission results. Of 105 varieties and inbred lines of sugar beet tested, U. S. 75 gave best results. Infection increased with duration of preconditioning period in the dark or reduced light up to 5–6 days, after which time the inoculated leaves were likely to die before symptoms appeared. Juices from *Chenopodium murale*, from New Zealand spinach, and from sugar-beet plants that showed marked symptoms, appeared to be the best inocula. Juice from *C. amaranticolor* was consistently poor. Some strains of the virus were transmitted more readily than others. Addition of sodium sulphite to the inoculum appeared to have no beneficial influence in any concentration. Low concentrations of KH_2PO_4 in the inoculum may have increased infection slightly.

For many years, the yellows virus of sugar beet (*Beta vulgaris* L.) was considered nontransmissible by mechanical inoculation. However, in 1949, Kassanis (6) reported transmission by rubbing juice from diseased plants over the surface of sugar-beet leaves to which an abrasive had been applied. He reported that a small number of the inoculated plants developed local lesions, and that only a small percentage of plants showing local lesions later developed systemic symptoms. He found also that success in mechanical transmission was increased when the test plants were given a conditioning period in the dark prior to inoculation. Watson (7), using a technique similar to that employed by Kassanis, reported good results in cross-protection tests with related and unrelated beet yellows viruses.

In spite of the above-mentioned reports, investigators from other European countries apparently have not been successful in transmitting the yellows virus mechanically. The only other record of mechanical transmission of this virus is that by Coons (3) who, working with virus yellows as it occurs in the United States, reported systemic infection in a relatively high percentage of inoculated plants. No local lesions were observed.

During the course of investigations carried out at Riverside, California, with the virus causing yellowing of sugar beet in the Western United States, tests on mechanical transmission were started by the writers. From April to August 1952, many unsuccessful attempts were made, and over 3000 plants of sugar beet and other hosts were inoculated mechanically with virus yellows before transmission was obtained. Later, from September 1952 to January 1953, fairly good transmission was achieved. The present paper reports the results and the experience gained in tests that involved the mechanical inoculation of over 10,000 plants.

SYMPTOMS INDUCED BY MECHANICAL INOCULATION.—Of more than 50 species of plants inoculated with juice from diseased tissue, only sugar beet and sowbane (*Chenopodium murale* L.) showed both local lesions and systemic infection. Local lesions were induced on 3 other species of *Beta* and on *Chenopodium album* L.

Sugar beet.—Under greenhouse conditions at Riverside, virus yellows induces the appearance of pinpoint local lesions, often necrotic, in sugar-beet leaves of suitable varieties 10–20 days after inoculation (Fig. 1, A). The lesions are roundish and usually less than 1 mm wide, with or without a light central spot. In early stages the lesions may be slightly raised, especially on the under side of the leaf; they also may appear as a central spot surrounded by a translucent halo. In some plants the lesions are distinct from the surrounding tissues, whereas in others it is necessary to observe the leaves carefully to detect them. In most cases the lesions are more noticeable on the dorsal than on the ventral side of the leaves, because of the lighter color of the former. Reddish lesions were observed on sugar-beet varieties that have a tendency to form red pigment.

The number of lesions varied considerably among inoculated plants of the same variety and among leaves of a single plant. Some leaves showed more than 100 lesions and others none. Very often a general yellowing of inoculated leaves followed the appearance of local lesions and part or the whole leaf withered and died.

Systemic invasion of the plant did not necessarily follow the appearance of local lesions. The plants with local lesions that developed systemic symptoms in different tests varied from 10–85 per cent. These systemic symptoms did not differ from those induced by inoculation with a vector, *Myzus persicae* Sulz., and

[1] Accepted for publication December 14, 1954.

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consisted of vein clearing or vein etching on young leaves, followed by yellowing as the leaves aged. Virus from some sources induced vein necrosis (Fig. 1, C), followed often by death of leaves that showed this type

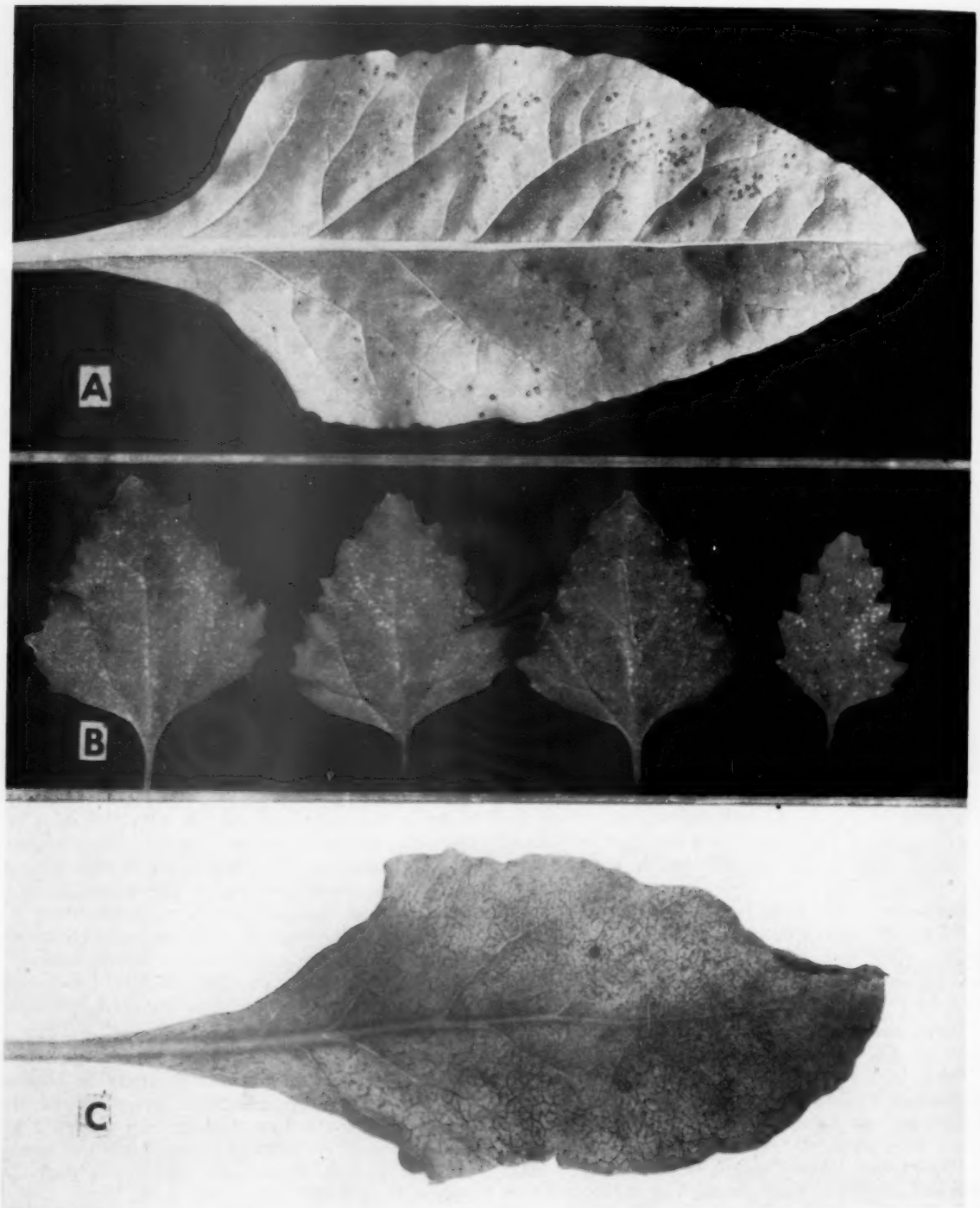

FIG. 1. Leaves showing the effects of virus yellows. A) Sugar beet leaf with typical local lesions produced following inoculation with juice from plants with virus yellows. B) Leaves of *Chenopodium murale* with local lesions produced following inoculation with juice from plants with virus yellows. C) Type of vein necrosis produced frequently on older leaves of sugar beet by one of the virus selections that gave best results in mechanical inoculations. All leaves about natural size.

of symptom.

Sowbane.—This species was more difficult than sugar beet to infect mechanically. In a few cases, however, plants subjected to darkness for 4 days prior to inoculation with juice from diseased plants, developed local lesions followed by typical systemic symptoms. Local lesions formed on *C. murale* were larger than those on sugar beet. They were whitish, slightly irregular along the periphery, and depressed as with etched areas (Fig. 1, B). Systemic symptoms were similar to those on plants infected by means of the green peach aphid and consisted of vein etching preceded by rolling of leaves. In some of the tests, systemic symptoms developed in plants of *C. murale* on which local lesions were never apparent.

FACTORS INFLUENCING TRANSMISSION.—Success in mechanical transmission of the yellows virus seemed to depend on a number of factors, the most important of which were: variety of test plant, source of virus, virus strain used, and preconditioning of test plants in the dark.

Variety of test plant.—The choice of a suitable variety of test plant was an important factor in achieving mechanical transmission of the yellows virus. Sugar beet, *Chenopodium murale*, *C. amaranticolor* Coste & Reyn., and New Zealand spinach (*Tetragonia expansa* Murr.) are all susceptible to virus yellows when inoculations are made by means of aphids. These 4 species were tried repeatedly in mechanical transmission tests, with and without preconditioning in the dark. Sugar beet was easier to infect than *C. murale*, and in no case was transmission obtained with test plants of the other 2 species. In some other tests occasional transmission of the yellows virus to plants of *Beta macrocarpa* Guss., *B. maritima* L., and *B. procumbens* Chrs. Sm. was obtained by mechanical inoculation.

Varieties of sugar beet seem to differ appreciably in susceptibility to infection from mechanical inoculation with the yellows virus. In early tests, plants of the variety S.L. 742 were used, but it was found later that other varieties gave better results. The effect that variety may have on amount of infection is illustrated by the results of comparative tests of 3 varieties shown in Table 1. In these tests, U.S. 75 was slightly better than U.S. 22/3 and both were much better than S. L. 742.

Tests were made with a collection of 105 beet varieties and inbred lines of sugar beet in an attempt to discover types more consistently susceptible to infection by mechanical inoculation than those already used. Attempts were made also to find a more satisfactory local-lesion test plant among more than 50 species of plants, other than sugar beet, belonging to the *Chenopodiaceae* and related families. Results thus far have indicated the sugar-beet variety U.S. 75 has as high a degree of susceptibility to infection as any of the different kinds of plants tested.

Age of test plant.—Mechanical transmission of the yellows virus was not achieved when inoculation was performed on the cotyledons of very young beet seedlings. Sugar-beet seedlings in the 4- to 5-leaf stage gave best results under the conditions of the experiments. When fully developed leaves of older plants were inoculated, they developed as many local lesions as leaves of younger plants, but systemic invasion occurred more often with younger plants.

Sugar-beet leaves of the same plant apparently are quite different in their physiological tone and this may be one of the reasons why the number of local lesions induced by mechanical inoculation with virus yellows varies so much from leaf to leaf. A technique similar to that of Holmes (5) was used in an attempt to minimize differences among leaves of the same plant. Apical growth of test plants was cut back 10–15 days before inoculation and the axillary growth trimmed when necessary. At the time of inoculation the younger leaves left on the plant were fully expanded and looked almost the same as the older leaves. When such plants were inoculated after a period in the dark, there was more consistency in the number of local lesions formed in the leaves of the same plant than in leaves of comparable nonpruned plants.

Conditioning period in the dark.—Both Kassanis (6) and Watson (7) reported that good results in transmission of the yellows virus by mechanical inoculation were obtained only on plants subjected to a few days of darkness prior to inoculation. The same observation was made at Riverside. Inoculated test plants not preconditioned in the dark showed local lesions only occasionally and systemic symptoms were produced even more rarely. Results of a few comparisons of the influence of duration of period of dark treatment on the susceptibility of plants to infection

TABLE 1.—*Reaction of 3 sugar-beet varieties to infection by juice inoculation*

Test no.	Percentage of plants of the indicated sugar beet variety infected of 20 inoculated		
	U.S. 75	U.S. 22/3	S.L. 742
1[a]	20	15	5
2[b]	65	40	20
3[b]	60	55	20

[a] Test plants kept in the dark for 4 days prior to inoculation.

[b] Test plants kept in the dark for 5 days prior to inoculation.

TABLE 2.—*Influence of duration of period in darkness on susceptibility of sugar-beet seedlings to infection by juice inoculation*

Test no.	Number of plants inoculated	Percentage of plants infected when inoculated after indicated number of days in dark						
		0	1	2	3	4	5	6
1	20	10.0	10.0	15.0	20.0	20.0	10.0	....
2	20	0.0	5.0	15.0	30.0	30.0	20.0	....
3	40	5.0	....	....	....	15.5	....	....
4	40	17.5	....	....	....	50.0	....	....
5	40	5.0	....	....	....	....	57.5	....
6	80	0.0	....	....	....	20.0	6.7	15.0

are shown in Table 2. These results indicate that the longer the test plants remained in the dark the more susceptible they became. There is, however, a practical limitation on duration of the treatment. Leaves of beet plants become progressively more brittle and more easily injured by rubbing as the period of treatment in darkness is prolonged. After 4 or 5 days of darkness, leaves that are inoculated tend to shrivel and die after the plants are returned to the light.

Complete darkness does not seem necessary to condition beet plants. A comparison was made between plants that had been preconditioned in almost total darkness and plants that had been kept in reduced light under a greenhouse bench. When the 2 types of plants were inoculated with the yellows virus, the number of local lesions obtained on plants subjected to reduced light was slightly, but not significantly, higher than on the plants subjected to total darkness.

Source of inoculum.—The kind of plants from which inoculum is derived appears to be one of the major factors in success in mechanical transmission of the yellows virus. For instance, no infection resulted in the many attempts made to infect test plants with inoculum obtained from sugar-beet field plants affected by virus yellows. Inoculum prepared from leaves from greenhouse plants that showed early symptoms of vein clearing or vein etching was not so effective as inoculum from leaves that had shown etching for some time or that were showing yellowing. This last result agrees with that obtained by European investigators (6, 7).

There seems to be a considerable difference in virus activity among inocula taken from different species of susceptible plants. In one test, inocula from *Chenopodium murale* and from New Zealand spinach were compared with 3 sources of inoculum from sugar beet. The 5 inocula were tested by the half-leaf method on adult beet plants, the growing point of which had been cut back about 12 days before inoculation. In addition, the plants were conditioned in the dark for 4 days. A total of 45 plants with from 4–6 leaves a plant were inoculated. The lesion totals for the 3 sources of virus from sugar beet were 84, 1995, and 366; for inocula from *C. murale* and from New Zealand spinach the totals were 6110 and 6740, respectively.

A summary of many other tests in which these various types of inocula were used is given in Table 3. In most instances, inoculum from leaves of *Chenopodium murale* showing etching symptoms gave better results than inoculum from sugar beet. New Zealand spinach also supplied a strong inoculum, whereas that from *C. amaranticolor* was consistently poor.

TABLE 3.—*Influence of source of inoculum on infection of sugar beet with the yellows virus by juice inoculation*

Source plant	Number of tests	Number of sugar beet seedlings		Percentage infection
		Inoculated	Infected	
Sugar beets	20	1201	319	26.6
N. Z. spinach	7	188	72	38.3
Chenopodium murale	6	187	128	68.5
C. amaranticolor	3	160	11	6.9
Spinach	1	20	3	15.0
Mixed samples	3	200	70	35.0

Virus strains.—As already mentioned, only 1 source of virus was available in the beginning of the present work. Later, new virus collections became available and were used in the tests. Although it is not possible to explain fully the lack of transmission in the early stages of the work and success in later experiments, it would not be surprising if this latter more successful result is attributable to the introduction of some of the new virus collections into the tests.

In several experiments virus selections that rather consistently caused marked vein necrosis of some of the systemically invaded leaves of U. S. 75 (Fig. 1, C), usually gave better results in mechanical transmission than virus from other sources.

Treatment of inoculum.—Sodium sulphite, an excellent preservative for viruses easily inactivated *in vitro* by oxidation (2, 4), apparently had no beneficial effect in the case of virus yellows when leaves from affected plants were ground in a mortar in the presence of solutions of varying concentrations of this salt. Phosphate buffer seemed to improve transmission in many instances, although in some of the successful tests good results were obtained also with inoculum extracted in the presence of water alone (Table 4).

The effect of buffer mixtures at various pH levels on transmission was studied in 2 tests. Limited results are not conclusive but they tend to indicate that 0.1 M phosphate buffer at pH 6, 7, and 8 was better than at pH 4 or 5.

Method of inoculation.—In the earlier phases of the work, inoculum was introduced into the plants by means of small needles inserted into the leaves and crowns of plants through drops of juice from diseased plants or through diseased tissue. Of more than 400 plants inoculated in this manner, none showed symptoms of virus yellows. In other tests, inoculum was introduced into the midribs and petioles of leaves and into the root by means of a small hypodermic needle. Sufficient inoculum was introduced to fill the intercel-

TABLE 4.—*Effect of different concentrations of phosphate buffer on transmission of yellows virus by juice inoculation*

Test no.	Number of plants[a] inoculated with each inoculum	Percentage of plants infected with juice extracted in a phosphate buffer system (pH 7) of the indicated molarity				
		0[b]	0.01	0.03	0.1	1.0
1	20	15.0	45.0	20.0	30.0	....
2	20	45.0	35.0	....	60.0	0.0
3	20	50.0	50.0	....	45.0	35.0
4	30	16.6	23.3	....	26.6	33.3
5	30	0.0	6.6	....	9.9	33.3

[a] Sugar-beet seedlings preconditioned in dark for 4 days prior to inoculation.
[b] Distilled water used instead of buffer.

lular spaces of an appreciable volume of the tissue of each plant with liquid. Of 50 plants inoculated, none showed evidence of infection.

Rubbing inoculum over the surface of leaves dusted with an abrasive was the only method by which infection was obtained. Variations in this technique, such as using different numbers of strokes in rubbing on the inoculum and performing the rubbing operation with a cheese-cloth pad or with the finger tips dipped in inoculum, were tried but showed no consistent differences. However, inoculation of the dorsal side of the leaf resulted in the production of more lesions than inoculation of the ventral side. In 1 test, for instance, in which one-half of each of 200 leaves was inoculated on the upper side and the other half of each leaf on the lower side, total lesion counts were 5763 and 9288 for the upper and lower sides, respectively. Usually, in routine tests, the inoculum was rubbed on both sides of the leaf.

Effect of environment.—During the summer months of 1952, when temperatures in the greenhouse were relatively high in spite of an operating air-cooling system, mechanical transmission of yellows virus was mostly negative. In the fall and winter months, when heating could be regulated so as to keep the temperature relatively low, fairly good results were obtained. As already pointed out, these differences in results may have been due to other factors, but environmental effects could have contributed to them. It should be noted, furthermore, that the sugar-beet seedlings used as test plants grew better under conditions of lower temperature.

Discussion.—The results presented in this paper indicate strongly that, although the yellows virus is transmissible by juice inoculation, transmission is conditioned by a number of factors not fully understood. Infection following mechanical inoculation may induce numerous local lesions but the production of local lesions is not always followed by systemic infection. The evidence that the local lesions observed were produced by the yellows virus is indirect. It consists principally of the following facts: 1) local lesion production was followed in a certain percentage of instances by systemic infection; 2) during the period of experimentation when no local lesions were being produced no systemic infection was observed; 3) local lesions were produced with juice from a number of different species of plants; 4) the time required for the appearance of local lesions, 10–20 days, is longer than that required for production of local lesions on beet by any other known virus and longer than that required for most other plant viruses; and 5) lesions observed were very similar to those produced in England (6, 7) following mechanical inoculation from yellows-infected sugar beet. There seems little reason to doubt, therefore, that the local lesions observed were caused by the yellows virus.

The reason for the long incubation period probably is to be found, in part at least, in the nature of the host plant. There is evidence that parenchyma cells of sugar-beet leaves may not be a favorable medium, in all stages of development and functioning, for increase of the yellows virus. Even in plants that have been infected for several weeks, virus content appears to vary markedly in different leaves of the same plant and some leaves seem never to attain a virus content sufficiently high to serve as a good source of inoculum in aphid transmission. Probably parenchyma cells will support increase of the yellows virus only when they attain a specific metabolic balance making for susceptibility. Such a condition might be present for only a short period in some cells and might never be attained in others. Under such conditions failure of the virus to become established in some cells and its slow development in those in which it does become established, might be expected.

This theory of differential susceptibility of leaves on the same plant to infection following mechanical inoculation is further supported by the fact that, in many instances, only 1 or 2 inoculated leaves on a plant showed local lesions, but usually those leaves that did produce local lesions produced them in abundance. The large number of lesions on a single leaf showed that the virus content of the inoculum was high. Therefore, virus must have been introduced into many cells of other inoculated leaves of the plant but encountered conditions unfavorable for increase.

The fact that unusually low percentages of inoculated plants that produced local lesions later came down with systemic infection probably indicates that the virus has difficulty in passing from parenchyma cells into the phloem and on to the growing point to produce systemic symptoms. This is by no means an unknown phenomenon among viruses of plants other than sugar beet, and with viruses that attack sugar beet it is a very common occurrence. For example, certain strains of cucumber mosaic virus (1), tobacco mosaic virus, spotted wilt virus, and tobacco streak virus produce numerous local lesions on sugar beet but none produces systemic infection, although all of these viruses have high invasive powers in other host plants.

The results and observations reported in this paper on mechanical transmission of sugar beet yellows virus in Western United States are similar to those obtained with sugar beet yellows virus in England, and constitute additional evidence of probable identity of the virus complexes causing yellowing of sugar beet in these 2 widely separated areas.

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PHYTOPATHOLOGICAL NOTES

A Method for Estimating Numbers of Motile Nematodes in Large Numbers of Soil Samples.[1] E. J. Anderson and I. Yanagihara. In the course of attempts to determine the effectiveness of nematicidal soil fumigants in relation to method of application and varying soil conditions, estimates of nematode populations in several hundred small samples in a short time appeared necessary. The sieving technique proved too slow and did not separate readily living from dead nematodes. The modified Baermann funnel technique, with glass funnels, required too much space and apparatus. To avoid these difficulties a further modification of the funnel technique was adopted.

Fig. 1 shows a diagram of the parts used. Conical paper cups of 12 oz. capacity (Lily Vee cold drink cup, Lily-Tulip Cup Corp.) were coated on the inner surface with hot paraffin applied with a small brush. For strength, a coated cup was then placed inside an uncoated cup with the point cut off and the 2 supported upright in a 12 oz. tin can. Aluminum or stainless steel 16-mesh window screen was shaped in a wooden template to form a shallow cup about 1 in. deep with a flat bottom and with sides to fit the angle of the paper cup. (Aluminum has the advantage of being shaped easily, and lacks springiness.) The diameter of the screen cup was such that the flat bottom, when in place, was about 1 in. below the rim of the paper cup. The cup was filled with tap water to about ½ in. below the rim and the screen was put in place. A soil sample, taken with a $2 \times 2 \times 2$ in. scoop, was placed in the center of a double sheet of facial cleansing tissue (Scotties, Scott Paper Co.), the edges of the tissue were brought up to form a bag, and the whole gently but quickly lowered onto the screen. When soil samples were collected in the field in small paper bags, identifying numbers were written near the top. As each sample was placed in the tissue for extraction, the portion of the bag bearing the number was torn off, inserted between the tin can and paper cup, and kept with the sample up to the time of examination. The water level was maintained at sufficient height to keep the sample saturated. After 4 days the cup was lifted from the supporting can and placed in an appropriate-sized ring over a laboratory sink. To permit removal of the nematodes which had accumulated in the water in the bottom of the cup, a long needle was inserted between the wall of the cup and the screen so as to puncture the bottom of the cup without disturbing the soil sample. Twelve to 15 ml of the nematode suspension was drained into a 2 oz. liquor glass. Several trials, in which various quantities were drained, showed that practically all the nematodes were contained in the 10 ml first drained off. About 30 minutes were required for the nematodes to settle to the bottom of the glass. The relative numbers and general types in the sample could then be determined under a dissecting microscope. Samples which required closer examination were poured into flat counting dishes.

The use of this method enabled the authors and 1 part-time assistant to take 800 soil samples from profiles of fumigation plots and to record estimates of

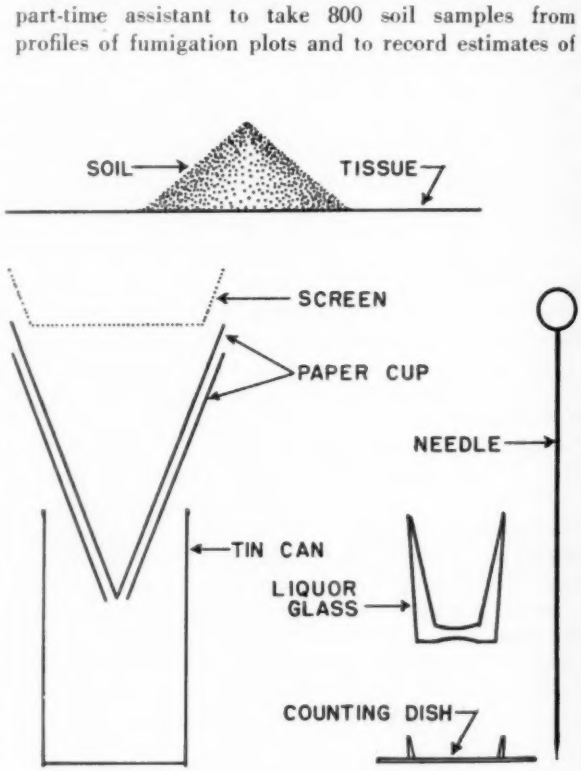

Fig. 1. Diagram of parts used in simple apparatus for extraction of nematodes from soil samples.

[1] Published with the approval of the Director as Technical Paper No. 229 of the Pineapple Research Institute of Hawaii.

nematodes in them in a total of 9 working days. Total area required for the simultaneous extraction of 800 samples was about 150 sq. ft. During extraction the assembly can be set on the floor, bench or shelf and can be moved readily without damage.

Molded Syracuse watch glasses were unsatisfactory for use as counting dishes because of strains and uneven surfaces. The lower side of a double-width microscope slide was scribed to give squares of a size such that an entire square could be seen in the field of the dissecting microscope. One end of the slide was ground so that numbers could be written on it with a lead pencil. The top $\frac{3}{8}$ in. of a liquor glass was then cut off and cemented to the marked slide immediately above the scribed area. This formed a counting dish of good optical characteristics and provided a means of estimating rapidly the number of nematodes. If care is taken to obtain a reasonably uniform distribution of the sample in the dish, the individuals in only a few squares selected at random need to be counted. The nematode suspension can be drained directly into such dishes, if desired.

Comparison of this method with the usual sieving technique indicated that it is several times more efficient in extraction of *Paratylenchus minutus* even when a 325-mesh sieve was used. Adults of this species are very small, averaging less than $\frac{1}{2}$ mm in length and are easily lost in sieving, but being active, readily pass through the tissue used. Great variability among replicates was present regardless of method of extraction. The paper cup method is empirical and its usefulness appears to be limited to relative estimates of motile forms in large numbers of samples. For this purpose it is clean, cheap, rapid, relatively accurate and easily carried out.—Pineapple Research Institute of Hawaii, Honolulu, Hawaii.

The Efficiency of a Quantitative Spore Collector Using the Cyclone Method. JOSEPH M. OGAWA AND HARLEY ENGLISH. An instrument was constructed to determine the relative concentration of spores of *Sclerotinia fructicola* (Wint.) Rehm in the atmosphere of orchards. This instrument had to have the following qualities: 1) the ability to collect spores in a size range of approximately 8-14 by 12-21μ, 2) the capacity to handle a large measured volume of air, and 3) a reasonably high degree of efficiency.

Many devices have been described for sampling particulate matter (pollen, spores, and other contaminants) in the atmosphere. The technique employed by Tervet and Cherry[1] and Tervet[2] for the collection of dry spores seemed preferable because it suggested the capability of quantitative determinations of *S. fructicola* spores in large volumes of air, whereas limitations on volume intake or place of spore collection existed in the others. Information on the efficiency of cyclone dust collectors provided by ter Linden[3] was used in the construction of the apparatus. The inside dimensions of the instrument (Fig. 1) in mm were as follows:

[1] Tervet, I. W., and E. Cherry. 1950. A simple device for collection of fungus spores. U. S. Dept. Agr. Plant Dis. Rptr. 34: 238.

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[3] Linden, A. J. ter. 1949. Investigations into cyclone dust collectors. Inst. Mech. Engin. Proc. 160: 233-251.

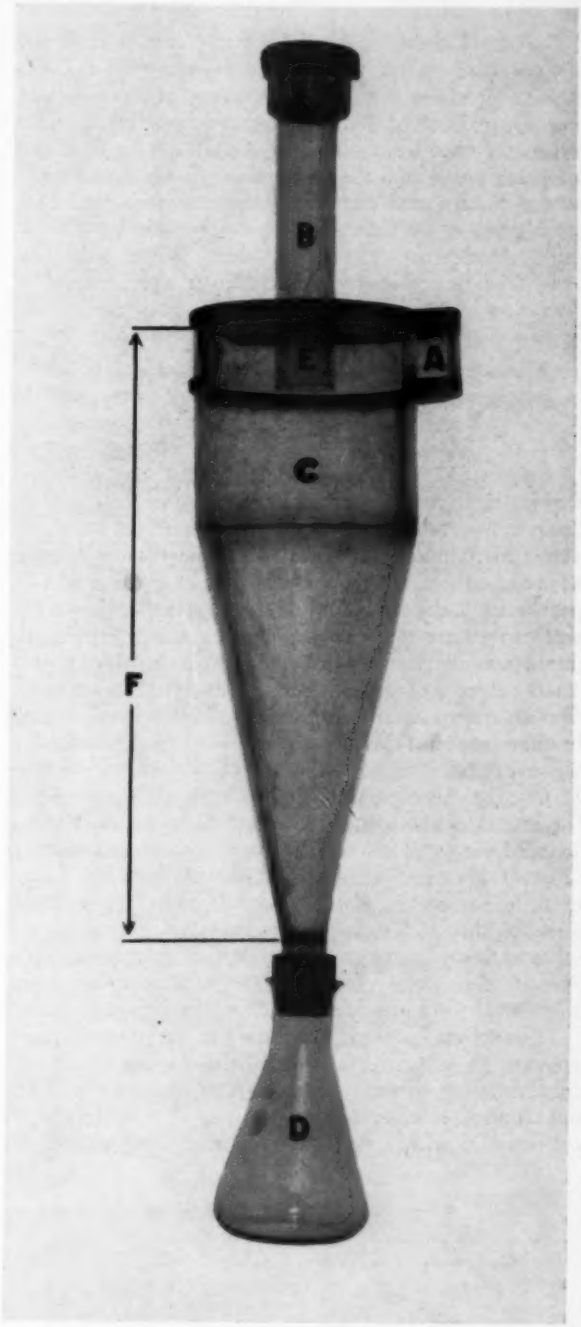

FIG. 1. The quantitative cyclone spore collector. Inlet A. Exhaust B. The spores are collected in the flask D.

height of cyclone (F) 262.5, height of cylindrical part of cyclone (C) 87.5, height of air inlet (A), 24.5, width of air inlet (A) 17.0, air exhaust diameter (B) 22.0, diameter of cyclone (C) 94.0, and length of exhaust pipe inside cyclone (E) 26.5. This apparatus was made of Lucite (a plastic manufactured by E. I. du Pont de Nemours and Co.). Air was drawn through the instrument by a vacuum pump operated by a portable gasoline engine. Air intake was measured by the attachment of a gas meter between the pump and the exhaust of the spore collector.

For determining the efficiency of the instrument, ether-washed spores of *Ustilago maydis* (DC.) Cda., measuring about 8 x 11μ, and spores of *Lycopodium* sp., approximately 30μ in diameter, were used. The weighed spores were drawn by the air stream from the weighing bottle into the instrument. A constant weight of the bottles and the bottles plus spores was first established at 24°C in a desiccator containing a saturated solution of calcium chloride. When a known weight of spores was drawn through the instrument operating at 8 cu. ft. of air/minute and reweighed, the efficiency of recovery was 97 per cent or greater.

Collection trials of spores of *S. fructicola* were made in peach orchards in which brown rot was present. The instrument was operated for a 2-hour period for each determination. The spores were collected either in a flask (Fig. 1, D) or on a vaseline coated cover slip which replaced the flask at the base of the cyclone. Spores from the flask were transferred to dilution plates containing potato-dextrose agar that had been adjusted to pH 2.2 by the addition of citric acid. It previously had been found that *S. fructicola* grew well on this medium whereas the growth rate of other fungi tested was markedly depressed. In the orchard tests, small colonies of several fungi, particularly species of *Cladosporium*, *Alternaria*, and *Penicillium* were found in abundance but there was no evidence of *S. fructicola*. Upon examination of the cover slips, however, a few *S. fructicola* spores were found. The possibility that the spores of the brown-rot organism might have been killed during collection was considered. It was shown, however, that a 24-hour period of operation had little or no effect on the viability of collected spores. It is possible that the ratio of conidia of *S. fructicola* to other collected spores was so low that colonies of this fungus were not obtained in the dilution plates, or, if present, were obscured by those of other organisms.

The cyclone spore collector used in the present study appears to offer great possibilities in the study of relative spore concentrations in the atmosphere. The quantitative efficiency is very high, the particle size collected is within the limits of most fungus spores, and the volume capacity is much higher than that of other instruments developed for spore collection. If the instrument were constructed of brass, it is probable that a higher volume capacity with the same range of efficiency would be achieved.—Department of Plant Pathology, University of California, Davis, California.

Variation in the Cultural Characteristics of Fusarium lini. C. S. VENKATARAM. Miller[1] has shown that cultures of the Fusarium that causes muskmelon wilt produce a fluffy mycelium on which mostly nonseptate conidia are borne in sparse numbers. This form is designated as the "wild type," and variants from it which are characterized by abundant sporulation have been attributed to mutations in culture.[2,3] Similar observations on *Fusarium lini* Bolley are reported here.

The original isolate of *F. lini* used in this study is designated as the "wild type" *sensu* Miller.[1] On most agar media it produced raised and fluffy colonies of white to pinkish mycelium. Conidia, mostly nonseptate and 1-septate, were scattered sparsely in the aerial mycelium. Twenty single spores from this "wild type" isolate were transferred to potato-sucrose agar slants. The cultural characters of these isolates in the second cultural generation were similar to those of the parent "wild type."

Of 120 monoconidial cultures in the third cultural generation, 119 produced the "wild" form, while 1 developed sporodochia after 2 weeks. In the fourth cultural generation, 60 single spore cultures made from 10 representative "wild" forms were like the parent "wild type." Correspondingly, 24 monoconidial cultures from the sporodochial variant gave only sporodochial lines, some of which produced pionnotes. None of the sporodochial variants reverted to the "wild type." Both "wild" and sporodochial lines retained their characteristics in 6 subsequent cultural generations. Origin of the sporodochial variant from the "wild type" apparently was due to mutation in culture. This is in line with previous observations on other Fusaria.[2,3]—Department of Plant Pathology and Botany, University of Minnesota, Institute of Agriculture, St. Paul 1, Minnesota.

[1] Miller, J. J. 1945. Studies on the Fusarium of muskmelon wilt. I. Pathogenic and cultural studies with particular reference to the cause and nature of variation in the causal organism. Canad. Jour. Res. Sect. C. 23: 16-43.

[2] Miller, J. J. 1946. Cultural and taxonomic studies on certain Fusaria. I. Mutation in culture. Canad. Jour. Res. Sect. C. 24: 188-212.

[3] Cormack, M. W. 1951. Variation in the cultural characteristics and pathogenicity of Fusarium avenaceum and F. arthrosporioides. Canad. Jour. Bot. 29: 32-45.

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NEWS

Editor: K. W. KREITLOW, Plant Industry Station, Beltsville, Md.

A nematology workshop was held at the United States Department of Agriculture's Golden Nematode Control Headquarters in Hicksville, Long Island, New York February 14-25, 1955 under the direction of Mr. Joseph F. Spears. This training course dealing with plant parasitic nematodes was sponsored by the Nematode Control Project of the Plant Pest Control Branch, Agricultural Research Service and brought together some 40 Federal and State employees in the New York area concerned with nematode regulatory problems.

The principal subject matter covered in this training course included laboratory techniques used in the preparation of microscopic mounts and the means of isolating nematodes from bulbs, plant tissues, and soil. These studies included anatomy, morphology and life cycles of nematodes. Since the personnel who attended this course were workers directly concerned with nematode regulatory problems, particular emphasis was placed on those groups of nematodes which have received special attention from a regulatory view point. Extra time was taken to study the genus *Heterodera* with such important species as *rostochiensis, tabacum, schachtii,* and *glycines.* Another laboratory session familiarized workers with the genus *Ditylenchus* containing the important species *destructor* and *dipsaci.* Additional time was allotted to either known or suspected agricultural pests such as species of *Meloidogyne, Belonolaimus, Trichodorus, Tylenchorhynchus, Pratylenchus* and *Xiphinema.* A full period was used to acquaint personnel with the nematode *Radopholus similis,* a potential threat to the citrus industry.

Instructors for the course were: A. L. Taylor, Section of Nematology of the U. S. Department of Agriculture, and Dr. J. N. Sasser, North Carolina State College. They were assisted by Dr. Victor Dropkin, Mr. George Fassuliotis of the Nematology Section and Mr. Daniel O. Betz, Golden Nematode Control Project. Many of the personnel attending the course were directly concerned with potato problems, therefore, eight hours of instructions was set aside for lectures and demonstrations concerning the principal varieties of potatoes and the diseases and insects that affect them. This phase of the instruction was under the direction of Cornell University instructors: Dr. Robert Dickey, Dr. Richard Sawyer and Dr. Maurie Semel.

A strawberry conference will be held at the U.S.D.A. Plant Industry Station, Beltsville, Maryland, on May 26 and 27. The first day will be devoted to breeding and production problems. The program for the second day includes root nematodes and their control, production of nematode-free stock and the nematode-black root complex.

The VIIth International Congress of Comparative Pathology will be held at the Institut d'Anatomie Pathologique, Lausanne, Switzerland, May 26-31, 1955. The program will include, in part, formal papers and discussions on viral infections transmitted to man by animals, pathologic and hygienic aspects of atmospheric pollution problems, and growth disturbances in comparative pathology including the human problem. Official languages of the Congress will be French, English, German, Italian, Spanish, and Russian. A technical exhibit will be provided and visits to scientific institutions will be made. Post Congress tours of 2 to 5 days in scenic Switzerland are planned. Titles and texts of communications must be submitted not later than May 1, 1955, to the General Secretary of the VIIth International Congress of Comparative Pathology, 19 rue César-Roux, Lausanne, Switzerland. Requests for the program and inquiries concerning registration should be addressed to the General Secretary, or to Lee M. Hutchins, Chairman, United States Section (Chief, Division of Forest Disease Research, Forest Service, U. S. Department of Agriculture, Washington 25, D. C.).

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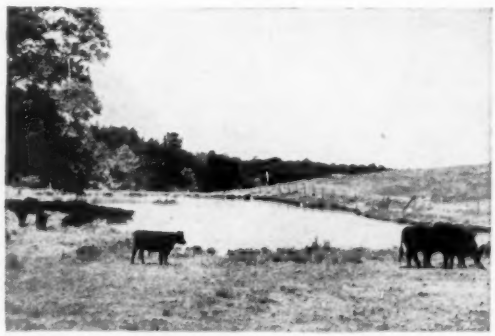

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